Cloning and Characterization of Goldfish Activin β_A Subunit and Regulation of Goldfish Gonadotropin Gene Expression by Activin

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Activin ($\beta_A\beta_A$, $\beta_A\beta_B$, $\beta_B\beta_B$) is a dimeric protein that belongs to the transforming growth factor-beta (TGF-β) superfamily. It plays important roles in a wide range of physiological and developmental processes. In fish, full-length cDNA for activin β_B subunit has been isolated from a number of species, but no such information is available for activin β_A. In the present study, a full-length cDNA coding for activin β_A subunit has been cloned from a goldfish brain and pituitary cDNA library. This represents the first for activin β_A in fish. Sequence analysis of goldfish activin β_A shows that this peptide is highly conserved across vertebrates. The mature region of goldfish activin β_A shares 81% amino acid sequence identity with that of humans. Messenger RNA of goldfish activin β_A is expressed in a variety of tissues including ovary, testis, brain and liver, suggesting a wide range of physiological roles for activin A in the goldfish. The identity of the cloned goldfish activin β_A was confirmed by expressing the protein in the Chinese hamster ovary (CHO) cells followed by detection of the specific activin activity in the culture medium using erythroid differentiation factor (EDF)-assay with F5-5 cells. Stable CHO cell lines producing high level of recombinant goldfish activin A were established and characterized.

It is well documented that the pituitary in teleosts produces two gonadotropins (GTHs), namely GTH-I (αIβ) and GTH-II (αIIβ), which may regulate different phases of the reproductive cycle. However, unlike in mammals, very little is known about the differential regulation of the two GTHs in fish. Using goldfish as a model, the present study demonstrates, for the first time, that activin, a protein factor that plays a critical role in the differential regulation of mammalian GTHs (FSH and LH), has opposite effects on GTH-Iβ and GTH-IIβ mRNA expression. Recombinant goldfish activin B stimulates GTH-Iβ but significantly suppresses GTH-IIβ mRNA levels in a dose-dependent manner in cultured goldfish pituitary cells. Administration of recombinant human follistatin, a specific activin binding protein, completely abolished the effects of activin, thus demonstrating the specificity of the activin activities. The novel opposite effects of activin on the two goldfish GTHs make goldfish a unique vertebrate model for activin studies. The present study not only contributes to our understanding of the mechanisms that control the temporal expression patterns of the two GTHs during the fish reproductive cycle, but also provides important information on the evolution of gonadotropin regulation in vertebrates.

摘要

激活素 ($\beta_A\beta_A$, $\beta_A\beta_B$, $\beta_B\beta_B$) 是由兩個亞基組成的蛋白質生長因子，其在結構和功能上屬於轉化生長因子 beta (TGF-β)家族。激活素在很多生理和發育過程中起著重要的作用。在魚類中，激活素β_B 亞基的全長互補去氧核醣核酸(cDNA)已經在數種真骨魚類中被克隆出來，可是相對的β_A 的資料還沒有在文獻中記載。在本研究中，我從金魚腦和腦下垂體 cDNA 文庫中克隆了激活素β_A 亞基的全長 cDNA，這在魚類中尚屬首次。cDNA 及其編碼的蛋白氨基酸序列分析表明，激活素β_A 亞基在脊椎動物進化中高度保守。比較金魚和人類的激活素β_A 亞基，其保守性高達百分之八十一。激活素β_A 亞基在金魚的卵巢，睾丸，腦和肝中均有表達，這表明了激活素 A 可能在一系列的生理反應中起著作用。爲了進一步研究激活素的生物學功能，我隨後利用克隆的金魚激活素β_A 亞基在中國倉鼠卵巢(Chinese hamster ovary, CHO)細胞系中表達和生產具有生物學活性的金魚激活素 A。經過一系列的細胞克隆，我分離得到了一些穩定的高產細胞株。實驗結果表明，這些細胞株產生的金魚激活素 A 具有良好的生物學活性，可以有效地誘導培養的小鼠白細胞系 F5-5 的分化。

與四足動物類似，魚類腦下垂體亦產生兩種不同的促性腺素(GTHs)，分別稱之爲 GTH-I(αIβ)和 GTH-II(αIIβ)。這兩 GTHs 在魚類生殖周期的不同階段可能起著不同的調控作用。但與哺乳類動物不

同，有關這兩種 GTHs 在魚類生殖周期中的差異性調控機理，目前尚未清楚。採用金魚作模型，我在本研究中首次證明了激活素對 GTH-Iβ和 GTH-IIβ基因的表達有相反的作用。重組金魚激活素 B 可以刺激 GTH-Iβ的表達，同時有效地降低 GTH-IIβ 的 mRNA 的水平，這些作用是劑量相關的。另外，激活素的結合蛋白 follistatin 可以完全消除激活素的作用，表明了激活素作用的特異性。激活素對金魚兩種 GTHs 所起的相反作用，使金魚成爲激活素研究的獨特模型。本研究不但增加了我們對 GTHs 在魚類生殖周期中不同階段的表達及其機制的了解，更爲脊椎動物中 GTH 調控的進化提供了重要的線索。

Acknowledgement

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Kwan Mei YAM

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Symbols and Abbreviations

Symbols

α	Alpha
β	Beta

Abbreviations

BES	*N*,*N*-bis[2-hydroxyethyl]-2-amino-ethanesulphonic acid
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CG	Chorionic gonadotropin
CHO	Chinese hamster ovary
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E_2	Oestradiol
EDF	Erythroid differentiation factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FS	Follistatin
FSH	Follicle-stimulating hormone
gf	Goldfish
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPH	Glycoprotein hormone

GTH	Gonadotropin
HBSS	Hank's balanced salt solution
HCG	Human chorionic gonadotropin
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
kb	Kilobase pairs
LH	Luteinizing hormone
MOPS	3-[*N*-morpholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
P	Progesterone
PCR	Polymerase chain reaction
rhFS	Recombinant human follistatin
rgfAct	Recombinant goldfish activin
rgfActA	Recombinant goldfish activin A
rgfActB	Recombinant goldfish activin B
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SSC	0.15 M NaCl/0.015 M Na_3·citrate, pH7.6
T	Testosterone
TGF	Transforming growth factor
TSH	Thyroid-stimulating hormone
w/o	without

Scientific Names

Common name	Scientific name
Black carp	*Mylopharyngodon piceus*
Chum salmon	*Oncorhynchus keta*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinius carpio*
European eel	*Anguilla anguilla*
Gilthead seabream	*Sparus aurata*
Goldfish	*Carassius auratus*
Japanese eel	*Anguilla japonica*
Killifish	*Fundulus heteroclitus*
Masu salmon	*Oncorhynchus masou*
Medaka	*Oryzias latipes*
Rainbow trout	*Oncorhynchus mykiss*
Red seabream	*Pagrus major*
Striped bass	*Morone saxatilis*
Tilapia	*Oreochromis niloticus*

Chapter 1

General Introduction

Gonadotropins (GTHs) from the pituitary are key players in vertebrate reproduction. They are involved in the regulation of gametogenesis, steroidogenesis, ovulation and spermiation, the essential events for the perpetuation of vertebrate life. In mammals, two GTHs have long been known to play critical roles in the reproductive processes. However, in fish, the duality of GTHs has only been confirmed recently. Increasing evidence shows that the two GTHs in fish are differentially regulated but the critical modulators behind remain to be elucidated. A potential candidate for this is activin, a recently identified protein that has attracted increasing attention. In this chapter, an overview of GTHs, and activin and its involvement in the differential regulation of GTHs would be given, followed by the objectives of the present study.

1.1 Gonadotropins (GTHs)

1.1.1 Structure

GTHs are heterodimers consisting of two subunits, an α subunit and a β subunit. In mammals, two GTHs are present, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH share the same α subunit, but each has its unique β subunit for exerting their specific biological activities. FSH and LH are glycosylated and classified under the family of glycoprotein hormones (GPH), which also includes pituitary thyroid-stimulating hormone (TSH) and placental chorionic gonadotropin (CG) (Fig. 1-1). It should be noted that all members of the GPH share a common α subunit, but each carries its own β subunit for their specific functions.

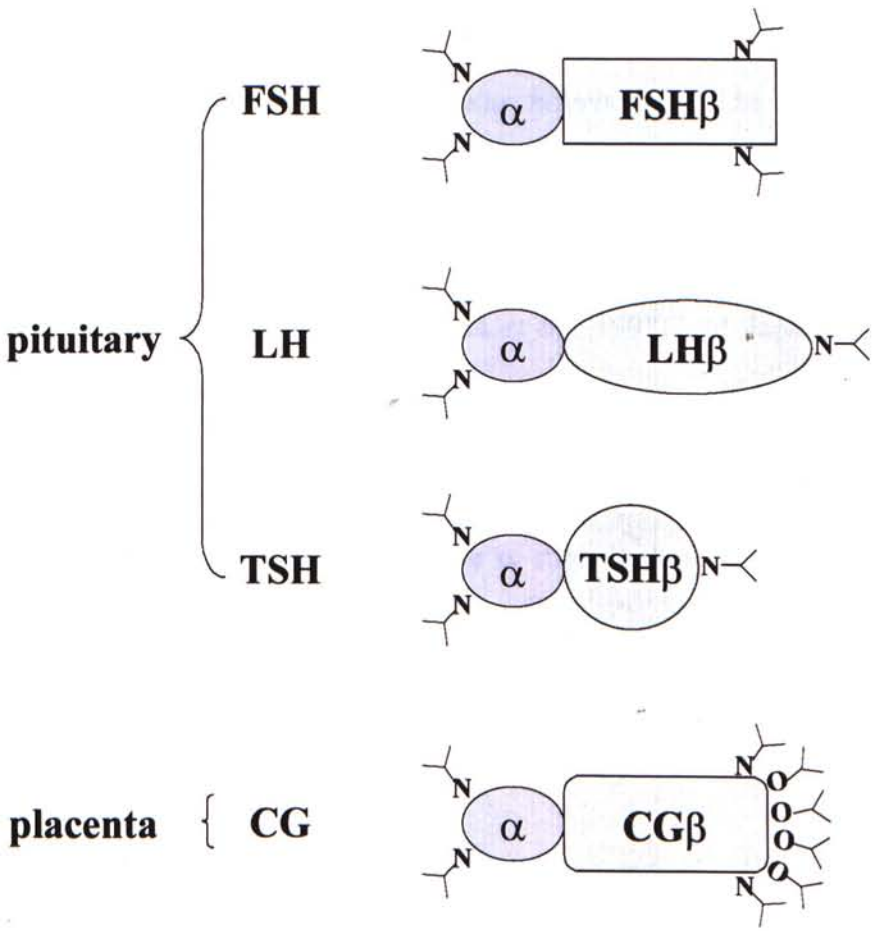

Fig. 1-1 Structure of glycoprotein hormones. (Modified from Albanese *et al.*, 1996)

In fish, the duality of GTHs has been confirmed by purification and characterization from natural sources in the chum salmon (*Oncorhynchus keta*) (Suzuki *et al.*, 1988a), coho *salmon (Oncorhynchus kisutch*) (Swanson *et al.*, 1991) and common carp (van der Kraak *et al.*, 1992). Molecular cloning of the GTH subunits in chum salmon (Sekine *et al.*, 1989), killifish (*Fundulus heteroclitus*) (Lin *et al.*, 1992), masu salmon (*Oncorhynchus masou*) (Kato *et al.*, 1993), striped bass (*Morone saxatilis*) (Hassin *et al.*, 1995), gilthead seabream (*Sparus aurata*) (Elizur *et al.*, 1996), goldfish (*Carassius auratus*) (Yoshiura *et al.*, 1997) and Japanese eel (*Anguilla japonica*) (Yoshiura *et al.*, 1999) also supports the duality of GTHs in fish. Similar to that in mammals, the two chemically distinct GTHs in fish, GTH-I and GTH-II, are also heterodimers sharing the same α subunit, but each has its own unique, function-specific β subunit (Fig. 1-2).

1.1.2 Function

The two GTHs have distinct functions in mammals. FSH mainly stimulates gametogenesis whereas LH specifically promotes steroidogenesis and gamete maturation. Though both FSH and LH are secreted from the anterior pituitary, they act on their specific receptors on different groups of cells. FSH acts on the Sertoli cells in the testis and the granulosa cells in the ovary. On the other hand, LH acts on the Leydig cells in the testis and the theca cells in the ovary. In the male, LH stimulates the Leydig cells to produce testosterone (T), which is reduced to 5α-reduced androgens in the Sertoli cells. This, together with FSH, promotes spermatogenesis (Fig. 1-3). In the female, LH stimulates T production in the theca cells. The T produced is then aromatized in the granulosa cells to form oestradiol

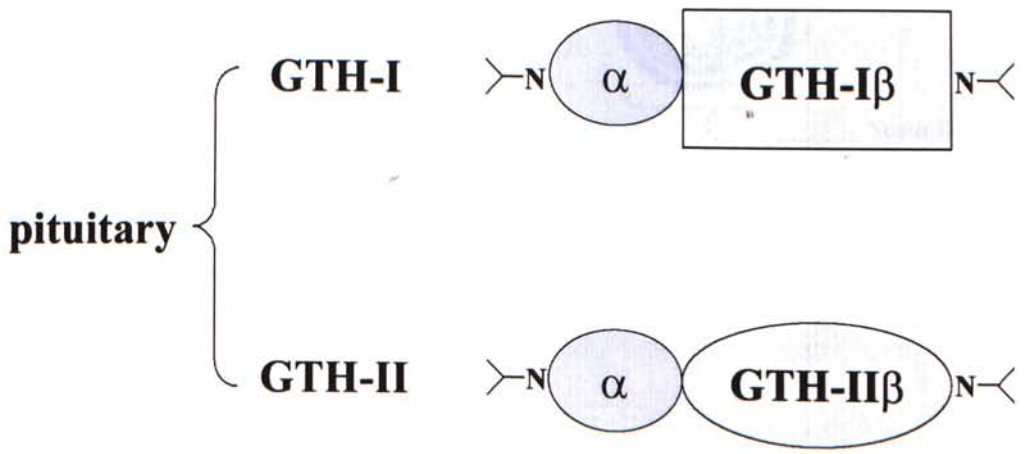

Fig. 1-2 Structure of fish GTHs. (Generalized from Elizur *et al.*, 1996; Hassin *et al.*, 1995; Kato *et al.*, 1993; Lin *et al.*, 1992; Sekine *et al.*, 1989; Suzuki *et al.*, 1988a; Swanson *et al.*, 1991; van der Kraak *et al.*, 1992; Yoshiura *et al.*, 1997, 1999)

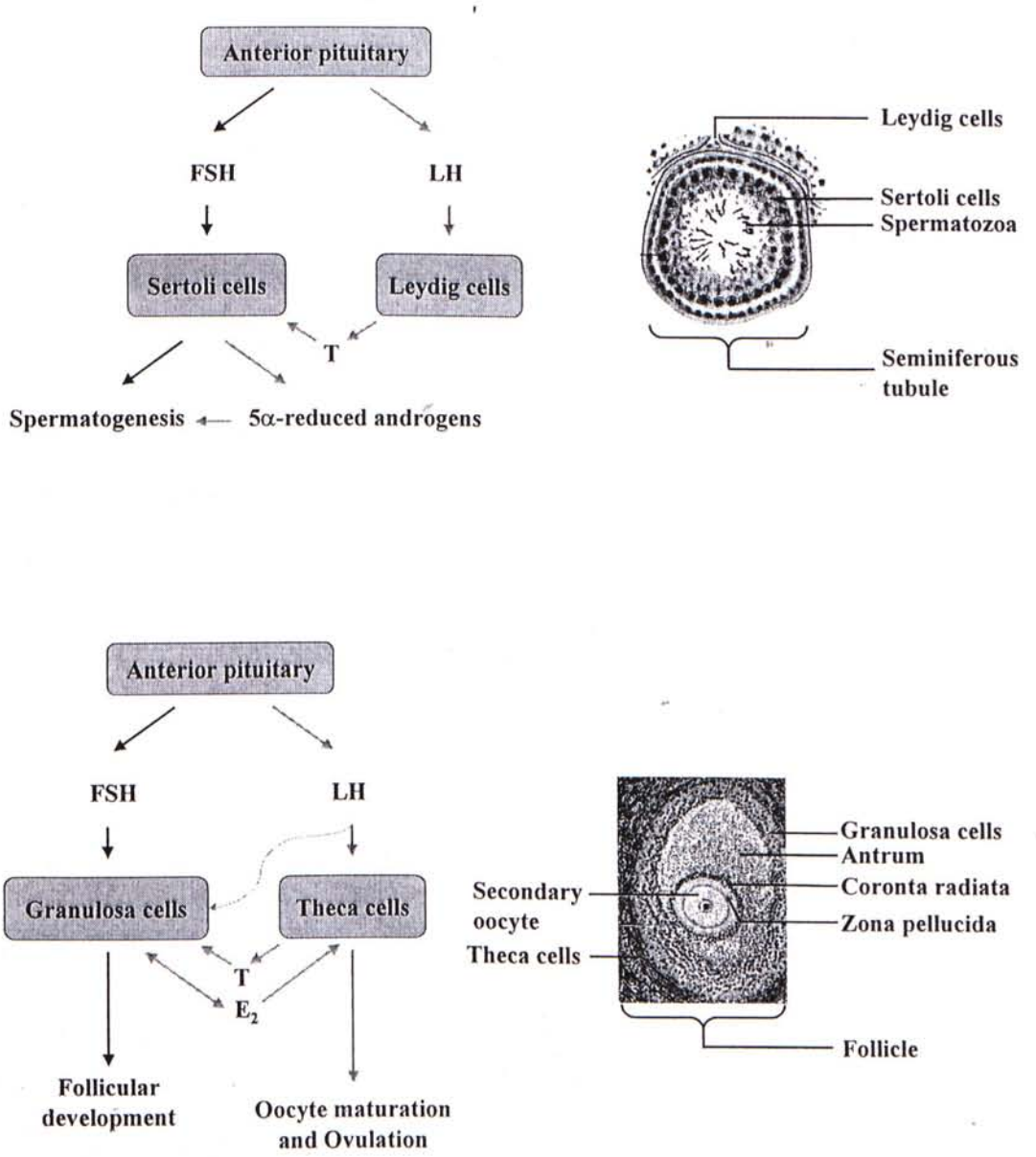

Fig. 1-3 Functions and mode of actions of FSH and LH in mammals. *Upper left panel*: GTH actions in the male. *Upper right panel*: a seminiferous tubule. *Lower left panel*: GTH actions in the female. *Lower right panel*: a follicle. (Models summarized from Albanese *et al.*, 1996; Schwartz 1995; pictures modified from Fox, 1993)

(E_2). This, together with FSH, stimulates follicular growth (Fig. 1-3). Besides, at certain stage of the reproductive cycle, E_2 exerts positive feedback effect, which leads to an LH surge and induces oocyte maturation and ovulation in the animal.

Though the structural duality of fish GTHs has been confirmed in a number of species as discussed earlier, the functional duality was not widely accepted until recently. This is because GTH-I and GTH-II share the same spectrum of biological activities causing stimulation of ovarian and testicular steroidogenesis and induction of oocyte final maturation *in vitro* (Suzuki *et al.*, 1988b; van der Kraak *et al.*, 1992). Not until some time after Swanson (1991) presented the seasonal profiles of GTH-I and GTH-II in the coho salmon (Fig. 1-4), was the functional duality of the two fish GTHs recognized. GTH-I level is high during vitellogenesis and is reduced before ovulation while the level of GTH-II remains low during the early stages of the reproductive cycle and increases dramatically before maturation. Similar profiles of GTH-Iβ and -IIβ mRNA have also been demonstrated in the rainbow trout and gilthead seabream (Weil *et al.*, 1995; Meiri *et al.*, 1995). These lines of evidence strongly suggest that GTH-I and GTH-II are probably involved in controlling different phases of the reproductive cycle like the mammalian FSH and LH, despite their similar activities *in vitro*. Though GTH actions in male fish are not well studied, it is known that GTHs act on the testis and stimulate Leydig cells to produce 11-ketotestosterone (11-KT), which in turn, acts on the Sertoli cells to stimulate spermatogenesis (Fig. 1-5) (Nagahama, 1994). In female fish, where more information is available, GTHs are known to act on the ovary and induce the production of T and 17α-hydroxyprogesterone in the theca cells. In the granulosa

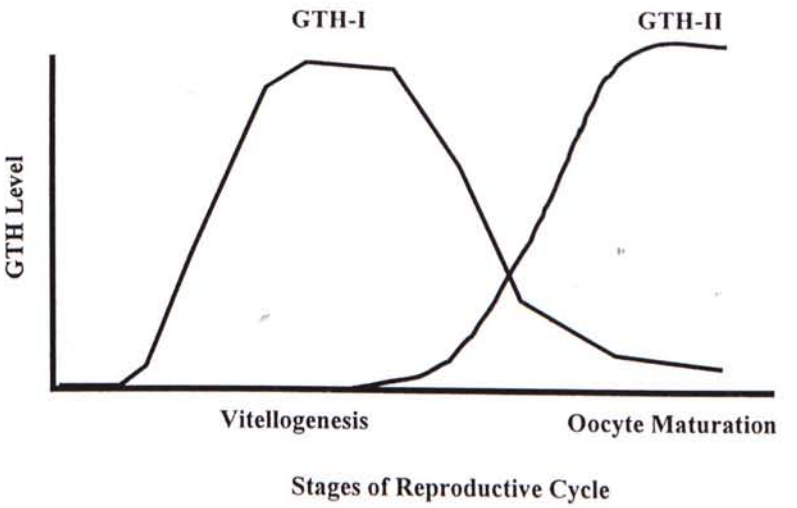

Fig. 1-4 Seasonal profiles of GTH-I and GTH-II in the coho salmon. (Redrawn from Swanson, 1991)

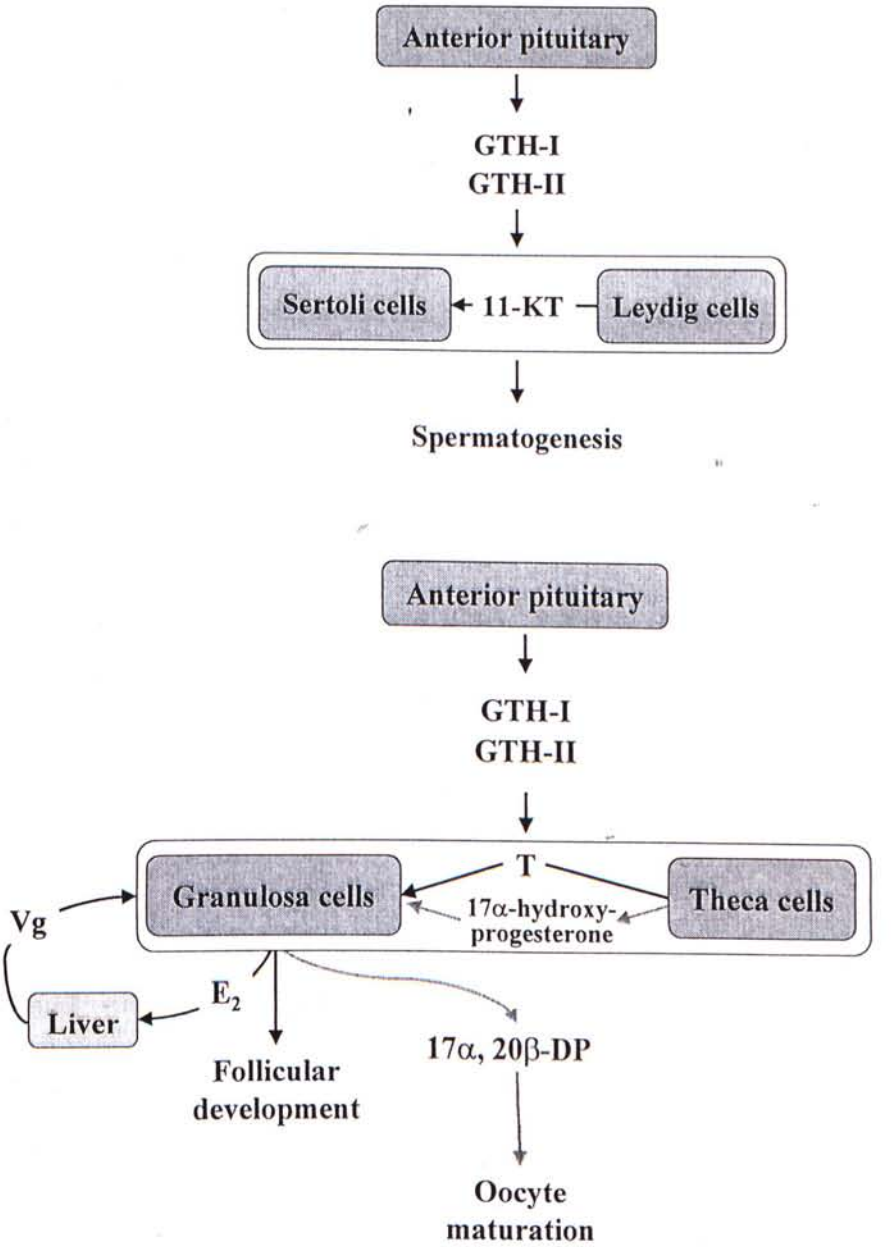

Fig. 1-5 Functions and mode of actions of GTH-I and GTH-II in fish. *Upper panel*: GTH actions in male fish. *Lower panel*: GTH actions in female fish. 17α, 20β-DP, 17α, 20β-dihydroxy-4-pregnen-3-one; 11-KT, 11-ketotestosterone; Vg, vitellogenin. (Generalized from Nagahama, 1987; 1994; Suzuki *et al.*, 1988b; Swanson, 1991; van der Kraak *et al.*, 1992)

cells, T is aromatized to E_2, which is transported to the liver to induce vitellogenin (Vg) production. Vg is incorporated into the oocytes, leading to rapid growth of the follicles. This process was reported to be stimulated by GTH-I (Tyler *et al.*, 1991). On the other hand, under the stimulation of GTH-II, the 17α-hydroxyprogesterone produced is converted to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), which induces the final oocyte maturation (Fig. 1-5) (Nagahama, 1987).

1.1.3 Regulation

GTHs are mainly regulated by three groups of factors: gonadotropin-releasing hormone (GnRH) from the hypothalamus, gonadal steroids and the recently identified activin family of growth factors (Fig. 1-6).

1.1.3.1 GnRH

GnRH stimulates both FSHβ and LHβ gene expression in mammals. In sheep, hypothalamic-pituitary disconnection reduces the expression of LHβ and FSHβ mRNA (Hamernik and Nett, 1988; Mercer *et al.*, 1989), while GnRH replacement restores the normal levels of both the subunits (Hamernik and Nett, 1988). In the rat, castration leads to an increase of the mRNA levels of LHβ and FSHβ subunits (Gharib *et al.*, 1986, 1987) while the addition of GnRH antagonist block the castration-induced increase of the LHβ mRNA levels (Lalloz *et al.*, 1988). Besides, GnRH is released from the hypothalamus in a pulsatile manner and its pulse frequency is regarded as an important factor in the differential regulation of FSH and LH (Besecke *et al.*, 1996). High pulse frequencies stimulate LH synthesis and secretion whereas slow frequencies stimulate FSH (Dalkin *et al.*, 1989; Jayes *et al.*, 1997; Spratt *et al.*, 1987). In fish, GnRH also stimulates both GTH-I and GTH-II.

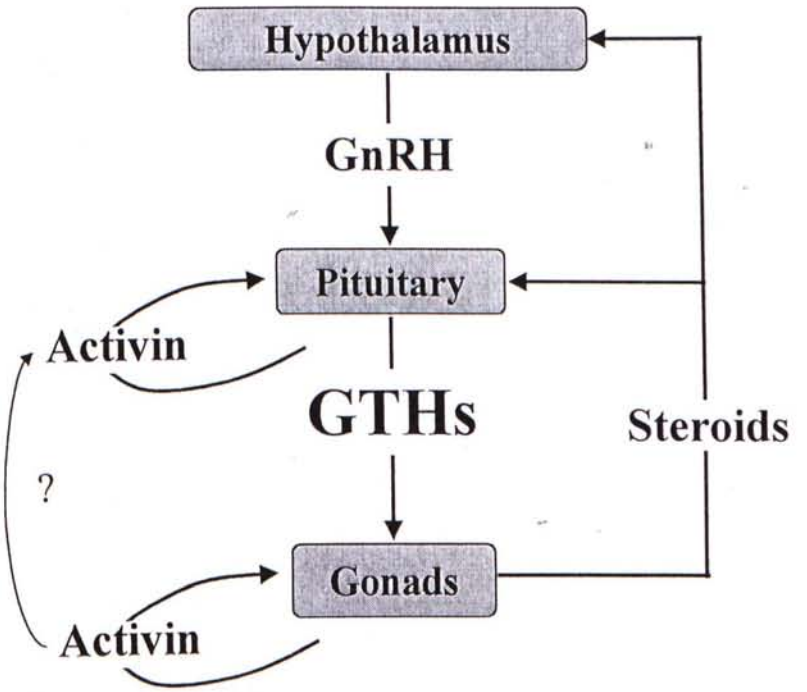

Fig. 1-6 The hypothalamo-pituitary-gonadal axis.

GnRH selectively stimulates GTH-I release in immature rainbow trout without affecting that of GTH-II. Whereas in the mature fish, the reverse occurs (Kawauchi *et al.*, 1989). In the goldfish and tilapia, GnRH stimulates GTH-II release and GTH-IIβ mRNA expression (Khakoo *et al.*, 1994; Melamed *et al.*, 1996). Similar effects were also suggested in the gilthead seabream (Gothilf *et al.*, 1997).

1.1.3.2 Steroids

Steroids from the gonads are under the influence of the pituitary GTHs. They, in turn exert feedback control to regulate the GTH levels. Classical castration experiments demonstrated immediate rise in LH and FSH secretion and β subunit mRNA levels (Abbot *et al.*, 1985; Corbani *et al.*, 1984; Gharib *et al.*, 1986; Kitahara *et al.*, 1991; Papavasiliou *et al.*, 1986). T and E_2 + P (progesterone) replacement in male and female, respectively, can abolish the castration-induced rise of GTHs (Dalkin *et al.*, 1993), demonstrating that steroids exert negative feedback action. On the other hand, T and E_2 can stimulate LH and FSH secretion and the respective β subunit mRNA levels in cultured rat pituitary cells (Gharib *et al.*, 1990; Lagace *et al.*, 1981; Miller and Wu, 1981). This indicates that steroids can also have positive effects directly at the pituitary level *in vitro*. In fish, both negative and positive feedback effects have been demonstrated in a number of species. Gonadoectomy or treatment with antisteroids increases the circulating levels of the GTHs in the goldfish (Kobayashi and Stacey, 1990), common carp (Breton *et al.*, 1975) and rainbow trout (Billard *et al.*, 1977; van Putten *et al.*, 1981). The increase is reversible following steroid replacement (Habibi *et al.*, 1989; Kobayashi and Stacey, 1990), indicating that steroids exert negative feedback at the hypothalamic or pituitary levels. Positive feedback actions have also been observed. T or E_2

treatment stimulates GTH-IIβ mRNA in immature and mature rainbow trout (Trinh *et al.*, 1986; Xiong *et al.*, 1994), the goldfish (Huggard *et al.*, 1996), black carp (Gur *et al.*, 1995), coho salmon (Dickey and Swanson, 1995), European eel (*Anguilla anguilla*) (Querat *et al.*, 1991), and maturing and regressed tilapia (Melamed *et al.*, 1997). However, T has no effect on GTH-IIβ mRNA in immature tilapia (Melamed *et al.*, 1997) and spawning rainbow trout (Xiong *et al.*, 1994). Besides, steroid implantation increases serum and pituitary GTH-II responses to GnRH applications in the goldfish (Trudeau *et al.*, 1991, 1993). Recently, T was shown to act directly on pituitary cells to increase GnRH-stimulated GTH-II release regardless of the states of gonadal maturation in the goldfish (Lo and Chang, 1998). On the other hand, T or E_2 treatment stimulates GTH-Iβ mRNA level in immature and maturing tilapia (Melamed *et al.*, 1997) while decreases that in the spawning fish (Melamed *et al.*, 1998). The treatment has no effect in regressed tilapia (Melamed *et al.*, 1998) and immature coho salmon (Dickey and Swanson, 1995). Therefore, it is evident that steroids play roles in the regulation of GTH levels. However, the nature of response heavily depends on the dose of steroids administered and the reproductive state of animals used, which determines the endogenous levels of circulating steroids (Habibi and Huggard, 1998; Melamed *et al.*, 1998).

1.1.3.3 Activin

Unlike GnRH and sex steroids, which affect both GTHs, activin specifically stimulates FSH secretion and FSHβ mRNA expression in mammals (Carroll *et al.*, 1989, 1991; Ling *et al.*, 1986b; Vale *et al.*, 1986; Weiss *et al.*, 1992, 1993, 1995) while having little effect on LH. In 1970s, researchers observed a secondary FSH surge for the recruitment of new follicles during the rat oestrus cycle (Fig. 1-7). This

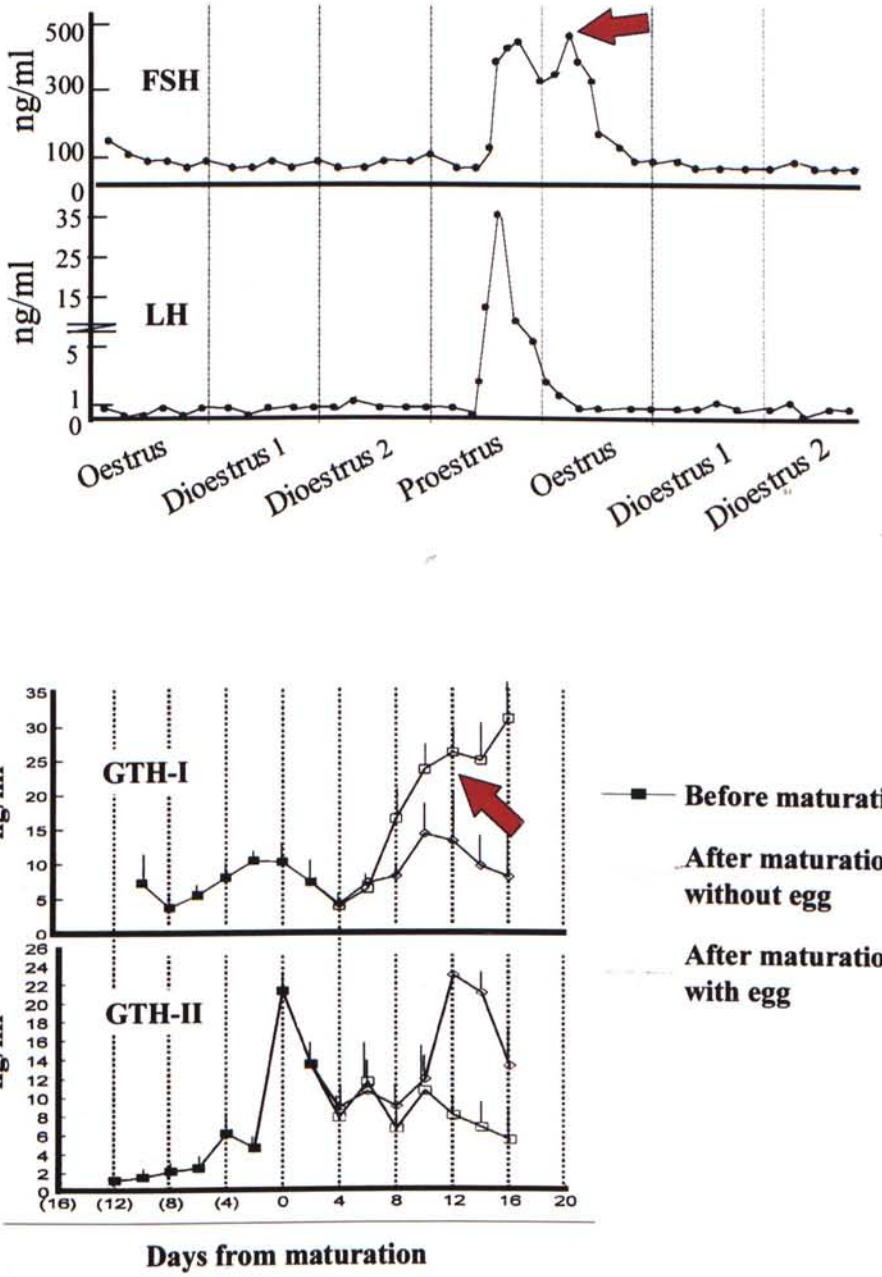

Fig. 1-7 GTH profiles. *Upper panel*: LH and FSH profiles during the rat oestrus cycle. The arrow indicates the second peak for FSH not observed for LH (Eckert *et al.*, 1988; Smith *et al.*, 1975). *Lower panel*: GTH-I and GTH-II profiles during maturation and ovulation in the rainbow trout. The arrow indicates the postovulatory surge of GTH-I after the release of eggs from the body cavity (Breton *et al.*, 1998).

secondary surge, not accompanied by LH, can be suppressed by a non-steroidal factor from porcine follicular fluid (Lorenzen *et al.*, 1978; Schwartz and Channing, 1977). Withdrawal of this non-steroidal gonadal factor, later characterized as inhibin, would unveil the action of activin, leading to the secondary rise in FSH level. The structure and functions of activin, particularly its regulation of GTHs will be discussed in the next section. In the rainbow trout, GTH-I and GTH-II were also differentially expressed during final maturation and ovulation (Fig. 1-7) as demonstrated by Breton *et al.* (1998). This differential regulation of GTHs depended heavily on whether the fish kept the eggs or not after ovulation, suggesting a possible role of gonadal factors in the differential regulation of GTH-I and GTH-II in fish. Breton *et al.* (1998) further speculated that activin family of growth factors might be involved. However, little information is available on this aspect.

1.2 Activin Family of Growth Factors

1.2.1 Stucture

Activin is a homo- or heterodimeric protein comprised of two β subunits linked by a disulfide bond. It shares the same β subunit with the distinct but related protein, inhibin, which is a heterodimer containing an α and a β subunit (Fig. 1-8). Five activin/inhibin β subunits have been reported to date, β_A, β_B (Ling *et al.*, 1986a, 1986b; Mason *et al.*, 1985, 1986, 1989; Tanimoto *et al.*, 1991; Vale *et al.*, 1986), β_C (Fang *et al.*, 1997; Htten *et al.*, 1995; Pohl and Schrewe, 1996); β_D (Oda *et al.*, 1995) and β_E; (Fang *et al.*, 1996). However, only three forms of activin [activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$) and activin B ($\beta_B\beta_B$)] have been isolated as dimeric proteins from natural sources (Fig. 1-9) (Fukui *et al.*, 1993; Ling *et al.*, 1985, 1986a, b; Nakamura *et al.*, 1992; Vale *et al.*, 1986, 1988; Ying, 1988). Activin belongs to

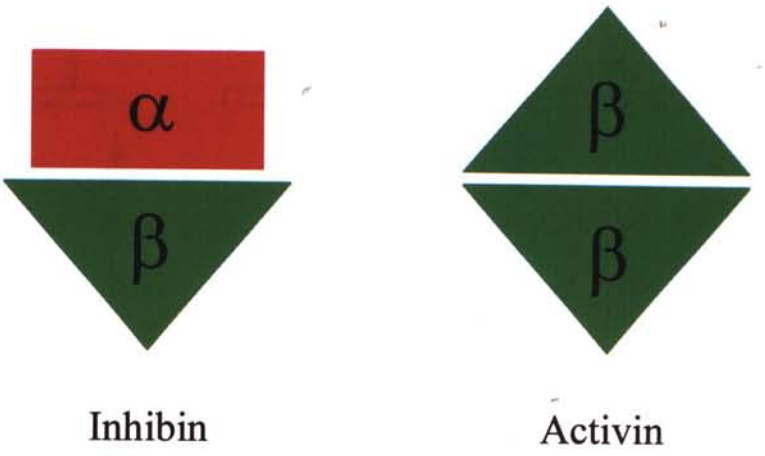

Fig. 1-8 Structure of inhibin and activin.

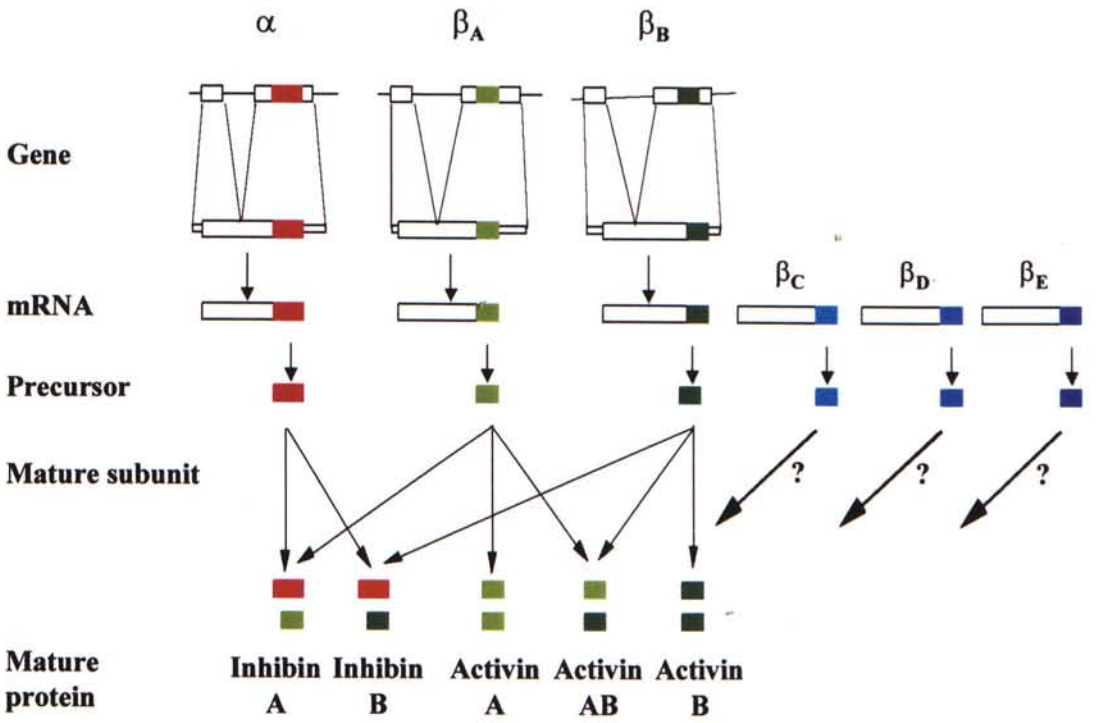

Fig. 1-9 Different activin/inhibin subunits form different mature proteins. (Generalized from Fang *et al.*, 1996, 1997; Htten *et al.*, 1995; Ling *et al.*, 1985, 1986a; Mason *et al.*, 1985, 1986, 1989; Oda *et al.*, 1995; Pohl and Schrewe, 1996; Tanimoto *et al.*, 1991)

the transforming growth factor-beta (TGF-β) superfamily of growth factors, which also includes TGF-β, inhibin, and bone morphogenetic proteins (BMPs). Like other family members, activin is only active as dimers, with the two chains linked by a single disulfide bond. Nine cysteine residues are conserved in the mature peptide. Apart from the one used for interchain bonding, all the others are used for intrachain disulfide bonds (Fig. 1-10) as predicted from X-ray crystallography (Daopin *et al.*, 1992). In fish, activin β_A and β_B subunits have been demonstrated in the goldfish (Ge and Peter, 1994; Ge *et al.*, 1993a, b; 1997a, b), zebrafish (Wittbrodt and Rosa, 1994), rainbow trout (Tada *et al.*, 1998) and Japanese eel (Nagahama, 1994) by immunocytochemistry or molecular cloning. Full-length cDNAs for activin β_B have also been isolated from the above species except the rainbow trout. However, no full-length sequence of activin β_A cDNA has yet been reported in any fish species.

1.2.2 Function

Activin, like other members of the superfamily, is a multifunctional peptide that has wide tissue distribution (Meunier *et al.*, 1988). It controls proliferation, differentiation and other functions in many cell types. In the pituitary, activin acts as a local autocrine factor to stimulate FSH production (Corrigan *et al.*, 1991; Weiss *et al.*, 1992). In the gonads, local activin promotes ovarian and testicular gametogenesis in a paracrine manner (Hsueh *et al.*, 1987; Mather *et al.*, 1990). Activin also regulates gonadal steroidogenesis (Hsueh *et al.*, 1987; Lejeune *et al.*, 1997; Sawetawan *et al.*, 1996) and induces ovarian apoptosis (Chun *et al.*, 1996). In the central nervous system, activin stimulates oxytocin release (Sawchenko *et al.*, 1988) and GnRH production (Gonazlez-Manchon *et al.*, 1991). Activin also induces

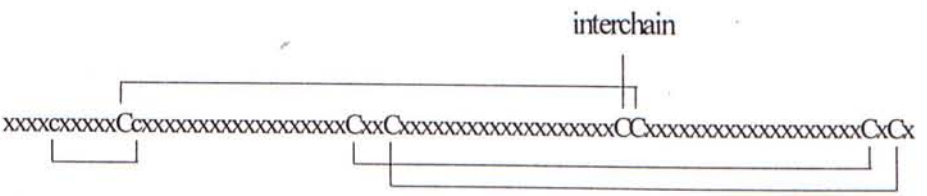

Fig. 1-10 Disulphide bond formation between cysteine residues in TGF-β family members. (Daopin *et al.*, 1992)

mesoderm and axis formation during development (Smith *et al.*, 1990; Thomsen *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1990) and stimulates erythrodifferentiation of mouse and human leukaemia cell lines (Eto *et al.*, 1987; Yu *et al.*, 1987).

Though activin has a vast array of actions in different target tissues, its effects on the differential regulation of FSH and LH have remained the focus of studies since its discovery and the results are well documented in mammals. Activin was first identified to exert stimulatory effects on pituitary FSH level, but not LH (Ling *et al.*, 1985, 1986a, b; Vale *et al.*, 1986). In cultured rat pituitary cells, activin selectively stimulates FSHβ mRNA expression at both the transcriptional and posttranscriptional levels (Carroll *et al.*, 1989; Weiss *et al.*, 1991, 1995) without affecting LHβ expression. In perifused rat pituitary cells, activin A increases both FSH secretion and FSHβ mRNA level (Weiss *et al.*, 1992, 1993). It should be noted that in most of these studies, the stimulatory effect of activin on FSH can be blocked by both inhibin, a natural antagonist of activin, and follistain, an activin-binding protein which can neutralize the bioactivity of activin (Hashimoto *et al.*, 1997; Kogawa *et al.*, 1991; Nakamura *et al.*, 1990) (Fig. 1-11).

Activin interacts with two different types of receptors, type I and type II, both of which are required to generate a transmembrane signal (Willis *et al.*, 1996). To date, two type I (ActRIA, ActRIB) and two type II (ActRII, ActRIIB) activin receptors have been identified (Attisano *et al.*, 1992, 1993; Donaldson *et al.*, 1992; Kondo *et al.*, 1996; Mathews and Vale, 1991; Mathews *et al.*, 1992; Shinozaki *et al.*, 1992). All of these contain an extracellular domain, a single transmembrane region and a

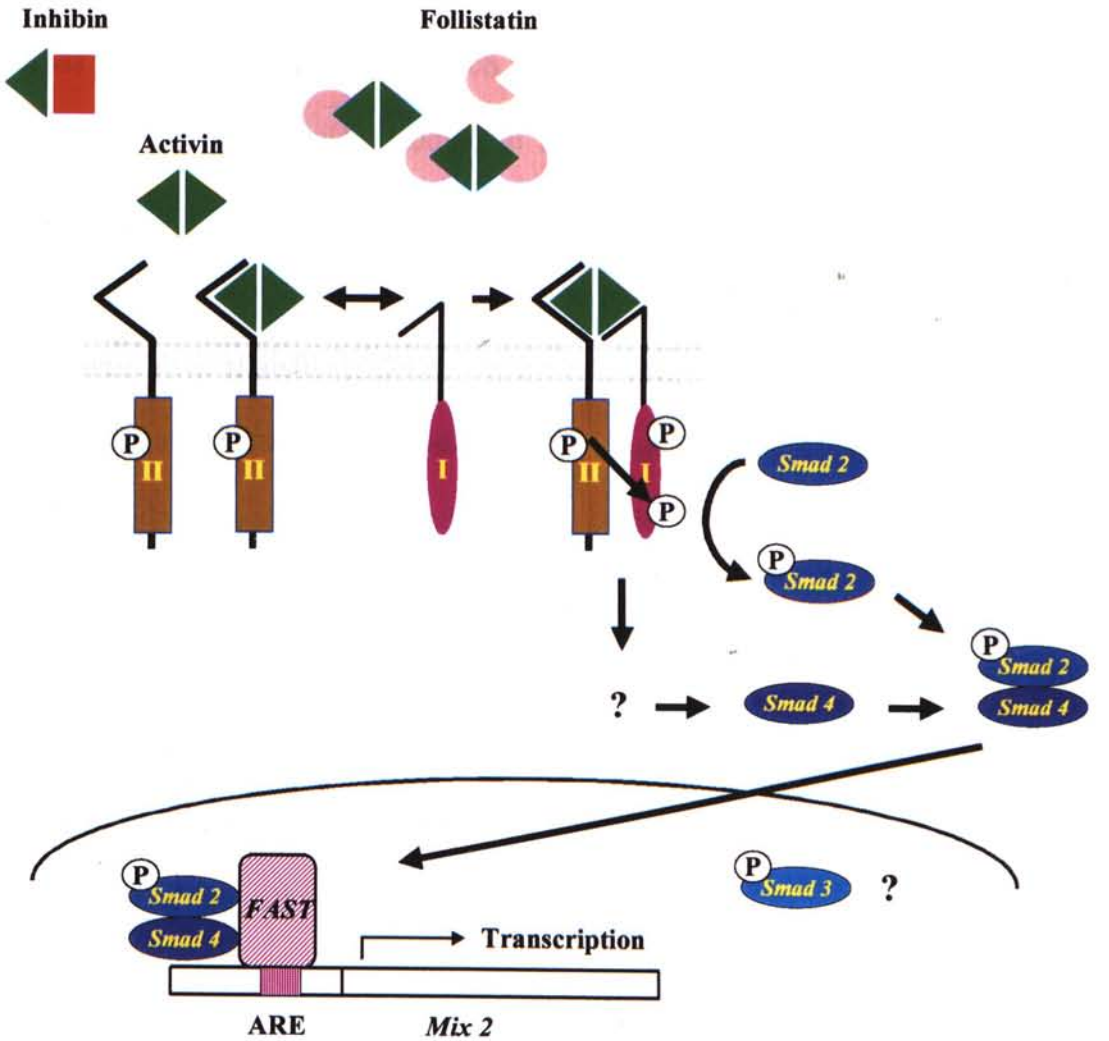

Fig. 1-11 Receptor binding and signal transduction of activin. (Modified from Lebrun *et al.*, 1997; Wrana *et al.*, 1994)

large intracellular domain with serine/threonine kinase activity (Fig. 1-11). The serine/threonine kinase domain in the type II receptor is constitutively phosphorylated (Mathews and Vale, 1993) whereas that in the type I receptor is phosphorylated only in the presence of a ligand. To initiate a response, activin first binds to its type II receptor, which stimulates the rapid recruitment of a type I receptor into the complex, leading to transphosphorylation and activation of the signal transduction pathway (Fig. 1-11) (Attisano *et al.*, 1993; Lebrun *et al.*, 1997; Willis *et al.*, 1996). In mammals, a group of proteins called *Smad*, has been found to mediate activin signalling. Study on the promoter of *Mix2* gene demonstrated that *Smad4* is associated with *Smad2* through oligomerization, then the complex is phosphorylated and translocated to the nucleus, where it interacts with the transcription factor *FAST* (forkhead activin signal transducer-I) at the activin-responsive element (ARE) to initiate transcription (Fig. 1-11) (Lebrun *et al.*, 1997). Besides, overexpression of *Smad3* in different cell lines leads to an increase in the activation of the activin-responsive promoter, thus suggests a role for *Smad3* in the activin-signalling pathway (Chen *et al.*, 1996).

In contrast to mammals, information on activin involvement in the differential regulation of fish GTHs is scanty though activin subunits have been demonstrated in different species, and porcine activin A has been shown to stimulate goldfish GTH-II release in perifused pituitary fragments (Ge *et al.*, 1992). Due to the limited supply of activin and the lack of specific radioimmunoassay (RIA) for GTH-I, particularly in cyprinids including the goldfish, studies on the differential regulation of the two GTHs have been hampered at the secretion level. Nevertheless, cDNAs for goldfish GTH-Iβ and -IIβ subunits have been cloned recently (Yoshiura *et al.*, 1997) and

recombinant goldfish activin B is now being produced in our laboratory (Ge *et al.*, to be submitted). These make possible the study of the differential regulation of GTH-I and GTH-II by activin in the goldfish at the transcriptional level.

1.3 Objectives

The present study aims at cloning the full-length activin β_A cDNA (Gactβ_A) from the goldfish, the first published sequence for full-length teleost activin β_A. The tissue distribution of the Gactβ_A will then be examined by Northern blot analysis (Chapter 2). The functional identity of Gactβ_A will be confirmed by expressing the protein in Chinese hamster ovary cells followed by detection of its specific activity by an EDF (erythroid differentiation factor)-assay. Stable cell lines that produce high level of bioactive activin A will be established for the recombinant production of goldfish activin A (Chapter 3). This, together with the recently produced recombinant activin B, will ease the problem of the limited supply of activin and the problems of specificity and efficacy arising from using heterologous mammalian molecules. Besides, this would make goldfish the only non-human vertebrate that has both recombinant activin A and B available, thus would allow detailed comparative studies on the two activins. Owing to the limited information available for the differential regulation of GTHs by activin in fish, the present study also attempts to investigate the potential role of activin in the regulation of the two GTH β subunit gene expression using a modified goldfish pituitary cell culture system (Chang *et al.*, 1990; Levavi-Sivan and Yaron, 1992). This represents the first of this kind in fish, and would provide important information on the evolution of GTH regulation in vertebrates (Chapter 4).

Throughout the study, goldfish will be used as the model due to several advantages offered by this species. Goldfish is small, easy to handle and readily available. It has long been used as a fish model for physiological studies. Besides, as a fish, it represents the lowest, but largest and most diverse group of vertebrates, thus would serve as an evolutionary reference for study of activin and GTHs. Moreover, goldfish belongs to the family of cyprinids, a group which is of aquacultural importance in the South China region; thus the study on the regulation of GTHs in the goldfish may provide invaluable information for manipulating fish reproduction in aquaculture.

Chapter 2

Cloning of Goldfish Activin β_A cDNA and the Expression of Its mRNA in Gonadal and Non-gonadal Tissues

2.1 Introduction

Activin ($\beta_A\beta_A$, $\beta_A\beta_B$ and $\beta_B\beta_B$) is a dimeric protein that belongs to the transforming growth factor-beta (TGF-β) superfamily of growth factors. It has widespread tissue distribution (Meunier *et al.,* 1988) and is involved in many important physiological and developmental processes including pituitary follicle-stimulating hormone (FSH) expression and secretion (Ling *et al.,* 1986b; Vale *et al.,* 1986; Weiss *et al.,* 1992, 1995), gonadal steroidogenesis (Hsueh *et al.,* 1987; Lejeune *et al.,* 1997; Sawetawan *et al.,* 1996) and gametogenesis (Mather *et al.,* 1990; Stock *et al.,* 1997), ovarian apoptosis (Chun *et al.,* 1996), oxytocin release (Sawchenko *et al.,* 1988), erythroid differentiation (Eto *et al.,* 1987; Yu *et al.,* 1987) and mesoderm induction (Smith *et al.,* 1990; van den Eijnden-Van Raaij *et al.,* 1990). In many cases, activin acts as a paracrine and autocrine factor regulating local physiological events (Chen, 1993).

In fish, activin has been identified and shown to play important roles in the regulation of reproduction and development. Porcine activin A stimulates pituitary growth hormone (GH) (Ge *et al.,* 1994) and gonadotropin-II (GTH II) (Ge *et al.,* 1992) release in the goldfish (*Carassius auratus*). Activin B induces mesoderm and axis formation in Japanese medaka (*Oryzias latipes*) (Wittbrodt and Rosa, 1994) and mediates gonadotropin-induced spermatogenesis in the Japanese eel *(Anguilla*

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japonica) (Nagahama, 1994). Recently, the genomic fragments of both activin β_A and β_B subunits have been cloned from the goldfish (Ge *et al.*, 1993b). Full-length cDNA of the β_B subunit has later been isolated from the goldfish (Ge *et al.*, 1997a), zebrafish *(Danio rerio)* (Wittbrodt and Rosa, 1994) and Japanese eel (Nagahama, 1994). These studies have provided invaluable information for studies of activin B in fish. In contrast to that in mammals, little information about activin A in fish is available, and no full-length cDNA sequence of activin β_A has yet been published in any fish species. Our previous studies have shown that when compared with that from other vertebrates, fish activin β_A subunit shows higher sequence variation than β_B (Ge *et al.*, 1993b, 1997a, b), suggesting functional difference between activin A and B. Cloning the full-length cDNA of activin β_A is therefore of particular importance for comparative studies on the two activins. However, our previous efforts to screen cDNA libraries for the goldfish activin β_A were not successful, probably due to the low abundance of the mRNA. The present study aims at isolating the full-length cDNA encoding activin β_A in the goldfish using polymerase chain reaction (PCR) and studying the tissue distribution of activin β_A mRNA. These studies not only provide information on the structural evolution of activin in vertebrates, but also allow us to further study the spatial and temporal pattern of its expression and regulation.

2.2 Materials and Methods

All chemicals were obtained from Sigma (St. Louis, MO) and restriction enzymes from Promega (Madison, WI) unless otherwise stated. Goldfish (*Carassius auratus*) were purchased from local market and maintained in a flow-through aquarium (1000

L) at 22°C on a 14L:10D photoperiod for at least one week before use. Fish were anaesthetized with tricaine methanesulphonate before handling.

2.2.1 Cloning of goldfish activin β_A cDNA

A goldfish brain and pituitary cDNA library constructed with lambda ZAP Express vector (Stratagene, La Jolla, CA) was used to isolate cDNA encoding activin β_A subunit. PCR was first used to amplify the 5' and 3' ends of the cDNA using primers based on our previously cloned β_A fragment (Ge *et al.*, 1993b). The amplified 5' and 3' ends were then mixed and subjected to extension by *Taq* DNA polymerase to yield the putative full-length cDNA as described below (Fig. 2-1).

2.2.1.1 Cloning of the 5' and 3' cDNA ends

Two rounds of PCR were carried out for each fragment. In the first round of PCR, primers M13-R (5'-AACAGCTATGACCATG-3')/ β_A-RT (5'-CATGTTCTGGATGTCTT-3') and β_A-U (5'-CAAAATCCGTGTCTGCTGTAA-3')/M13-20 (5'-GTAAAACGACGGCCAGT-3') were used to amplify the 5' and 3' ends, respectively, using the cDNA library as the templates. Whereas in the second round of PCR, T_3 (5'-ATTAACCCTCACTAAAG-3')/ β_A-L (5'-ACAACGAGGAGCAGAAGATC-3') and β_A-U-2 (5'-CCATGTGGC CAGCATCA-3')/T_7 (5'-AATACGACTCACTATAG-3') were used as nested primers and the first PCR products were used as the templates (Fig. 2-1). Both PCRs were carried out on Peltier Thermal Cycler 100 (MJ Research, Watertown, MA). Thirty cycles of reaction (template in 1X PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 mM each primer and 0.6 U *Taq* DNA polymerase in a final volume of 50 µl) were performed using a cycle profile of 94°C for 40 sec, 55°C

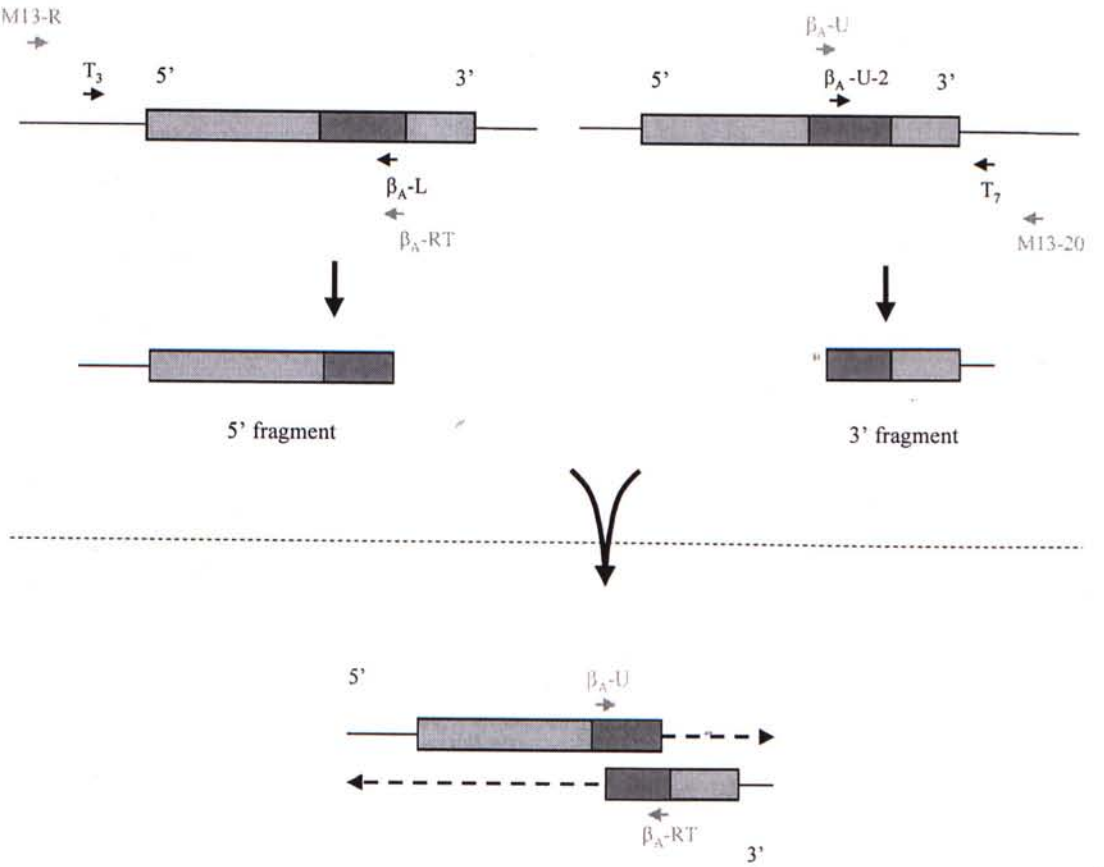


Fig. 2-1 Cloning strategy for activin β_A cDNA. *Upper panel*: Nested PCR strategy for amplifying the 5' and 3' fragments. The outer primers are indicated by grey arrows and the inner primers by black arrows. The hatched area represents the previously cloned genomic fragment. *Lower panel*: Extension of the 5' and 3' fragments to yield the putative full-length activin β_A cDNA. Primers β_A-U and β_A-RT, which only amplify the extension products (as indicated by arrows), were used to verify the identity of the putative full-length activin β_A cDNA.

for 30 sec and 72°C for 2 min after a 4-min denaturation at 94°C and followed by a final 5-min extension at 72°C. The PCR products were examined by electrophoresis in 1% agarose gel. The desired bands were excised, purified with GeneClean II (BI0101, Vista, CA) and ligated by T/A cloning into pBluescript II KS(+) (pKS) (Stratagene) at the *Eco*RV site. Several clones for both the 5' and 3' ends were analysed. The identity of the β_A clones was confirmed by PCR and the orientation of insert determined by restriction analysis with *Bst*XI, which has a unique site in the previously cloned fragment.

2.2.1.2 Extension of the 5' and 3' fragments

The 5' and 3' ends of the cDNA were mixed, trimmed by 5 U of T_4 DNA polymerase in a 50 µl reaction with 100 µM dNTPs, 0.1 mg/ml BSA in 1X Multicore Buffer (Promega) at 11°C for 2 hours and subjected to 15 cycles of extension using *Taq* DNA polymerase in standard PCR buffer, during which two fragments served as both templates and primers (Fig. 2-1). The extension products were cloned into pKS between the *Eco*RI and *Xho*I sites. This generated the construct pKS/Gactβ_A. The identity of the putative full-length cDNA (extension products) was confirmed by PCR using β_A-U and β_A-RT primers, which only amplify the extension products (Fig. 2-1).

2.2.2 Sequencing of the cDNA

2.2.2.1 Generation of pKS/Gactβ_A constructs with insert in different orientations

To ease the deletion procedure for the generation of overlapping subclones described in the next section, constructs with insert in different orientations were

prepared. This allowed a wider choice of restriction enzymes for linearization so that those with cutting sites within in the insert could be avoided. Briefly, the pKS/Gactβ_A construct was double-digested with *Eco*RI and *Xho*I. The protruding ends of the insert were filled-in using *Taq* DNA polymerase. These filled-in inserts were subcloned by T/A cloning into pKS at the *Eco*RV site, thereby generating pKS/Gactβ_A constructs with inserts in both orientations as determined by *Bst*XI restriction analysis.

2.2.2.2 Generation of overlapping subclones

The sequencing of the putative activin β_A cDNA was performed on a series of overlapping subclones generated by exonuclease III and mung bean nuclease deletion (Sambrook *et al.*, 1989). Briefly, two pKS/Gactβ_A constructs with the insert in different orientations were double-digested with *Hind*III and *Kpn*I to produce linearized constructs with 5' and 3' protruding ends, respectively. A 12 µl deletion cocktail [150 U exonuclease III (Gibco BRL) and 12 µg double-digested DNA in 1X exonuclease III buffer (Gibco BRL)] was incubated at 32°C. At 1.5 min interval, a fraction (2 µl) of the deletion cocktail was removed, mixed with 18 µl mung bean nuclease buffer in a 1.5 ml microtube and heat-inactivated at 70°C for 5 min to terminate the exonuclease activity. Mung bean nuclease (0.2 U in 2 µl) was added to each reaction and the mixture was incubated at 32°C for 30 min, and heat-inactivated at 70°C for 5 min. The reaction mixtures were electrophoresed in 1% agarose gel and the bands were excised and purified with GeneClean II. The purified deletion fragments were subjected to Klenow treatement [1.25 U Klenow fragment in 1X Klenow buffer] without dNTPs at 28°C for 5 min, followed by a

further 15-min-incubation in the presence of 1 mM each dNTPs. To monitor the reaction, a fraction of the reaction mixture was electrophoresed. The Klenow-treated deletion fragments were religated and transformed into competent XL1-Blue cells to generate the overlapping sublcones. These subclones were screened by PCR using T_3 and T_7 primers. A series of subclones with a size difference of about 300 bp between consecutive ones were chosen for sequencing.

2.2.2.3 Cycle sequencing

Both strands of the cDNA were sequenced. The sequencing reactions [4 μl of Terminator Ready Reaction Mix (Applied Biosystems, Foster City, CA), 1.6 μl of 1 μM T_3 primer and ~60 ng of deletion fragments in a total volume of 10 μl] were performed on Peltier Thermal Cycler 100 (MJ Research). Twenty-five cycles of the reaction were carried out with a profile of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The reactions were subjected to capillary electrophoresis on Genetic Analyzer 310 (Applied Biosystems).

2.2.2.4 Sequence analyses

Sequence analyses were performed using the computer program Genetyx and the identity of the clone (pKS/Gactβ_A) was confirmed by comparison with the GenBank database.

2.2.3 Isolation of total and messenger RNA

2.2.3.1 Isolation of total RNA

Total RNA was isolated from different goldfish tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Briefly, one hundred milligram of tissue was homogenized in 1 ml of TRI reagent and the homogenate was incubated at room temperature for 5 min. Then, two hundred microlitres of chloroform was added and the mixture was incubated at room temperature for 10 minutes. The aqueous layer was separated from the organic layer by centrifugation, and transferred to a fresh microtube. Half a millilitre of isopropanol and 0.5 µl of glycogen (20 mg/ml) were added for precipitation. After a 10-min-incubation at room temperature, the total RNA was pelleted, washed with 75% ethanol and dissolved in DEPC-treated water.

2.2.3.2 Isolation of messenger RNA

One milligram of the total RNA was used for the extraction of mRNA using the PolyATtract mRNA Isolation System III (Promega). Briefly, one milligram of total RNA in 500 µl of DEPC-treated water was denatured at 65°C for 10 min. Three microlitres of Biotinylated-Oligo(dT) Probe and 13 µl of 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) were added to the denatured RNA for annealing. The mixture was incubated at room temperature until completely cooled. The entire contents of the annealing reaction were added to a tube containing washed and resuspended SA-PMPs (Strepavidin-Paramagnetic Particles) in 100 µl of 0.5X SSC. The mixture was incubated at room temperature for 10 min. The SA-PMPs were captured using the Magnetic Stand and the supernatant was carefully removed. The particles were washed four times with 300 µl each of 0.1X SSC. After the final wash, the aqueous phase was removed as much as possible without disturbing the

SA-PMP particles. The mRNA was eluted twice with 100 μl and 150 μl, respectively, of DEPC-treated water.

2.2.4 Southern blot analysis

The cDNAs of goldfish activin β_A and β_B were separated from the plasmid vector by *Bam*Hl/*Hin*dIII and *Xho*I/*Not*I digestion, respectively. The digested plasmids (1 μg) were electrophoresed in 1% agarose gel, blotted onto positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) using VacuGene XL System (Pharmacia, Uppsala, Sweden), and UV-crosslinked at 150 mJ using GS GeneLinker (Bio-Rad, Hercules, CA). A full-length Gactβ_A cRNA probe was labelled with digoxigenin (DIG) by *in vitro* transcription using 1 μg linearized DNA, 1X DIG-RNA Labelling Mix (Boehringer Mannheim), 40 U T_3 RNA polymerase and 10 mM DTT in 1X Transcription Buffer. The nylon blot was hybridized overnight with the probe (25 ng/ml) at 50°C in hybridization solution containing 5X SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent (Boehringer Mannheim) and 50% formamide after a 2-hour-prehybridization at 50°C in hybridization solution without the probe. After hybridization, the blots were washed twice at room temperature with 2X SSC/0.1% SDS for 5 min each and twice at 65°C with 0.5X SSC/0.1% SDS and once with 0.1X SSC/0.1% SDS for 15 min each. Detection was performed using the Chemiluminescence Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, the membrane was blocked with blocking solution [1:10 w/v Blocking Reagent/maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5)] at room temperature for 1 hour after being equilibrated in washing buffer [maleic acid buffer, pH 7.5 and 0.3% w/v Tween 20]. The blocked membrane was incubated with anti-DIG antibodies

(1:10,000) at room temperature for 30 min, washed twice in washing buffer for 15 min each, and equilibrated in detection buffer (100 mM Tris-HCl and 100 mM NaCl, pH 9.5). Substrate solution (1:100 v/v CSPD/detection buffer) was applied to the membrane followed by exposure to the X-ray film.

2.2.5 Northern blot analysis

Three to seven micrograms of mRNA (isolated from 1 mg of total RNA) from different goldfish tissues were electrophoresed in 1.1% formaldehyde denaturing gel [1.1% agarose in 1X MOPS (40 mM MOPS, 10 mM NaOAc and 1 mM EDTA, pH 7.0) and 2.2 M formaldehyde], blotted onto positively charged nylon membrane, UV-crosslinked, hybridized and detected by chemiluminescence as described above.

2.2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Single strand cDNA was synthesized from 1.5 μg of total RNA using 100 U Superscript II reverse transcriptase (Gibco BRL) in a 10 μl reaction with 10 mM DTT, 0.5 mM dNTPs, 0.5 μg oligo-dT in 1X 1st Strand Buffer at 48°C for 1 hour. One twentieth of the RT reaction was used as the template for PCR amplification. To avoid genomic DNA contamination, two pairs of primers were designed to span a possible intron site according to the genomic structure of human activin β_A subunit (Tanimoto *et al.*, 1991) (Fig. 2-2). Two rounds of PCR were carried out. The first round of PCR used β_A-U(UTR)-2 (5'-ATCTTGGCAGCGGAGCAGAA-3') and β_A-L-2 (5'-CCCTTGTTGCTTTCTGTGGG-3') as the upper and lower primers, respectively. After the first PCR, the mixture was diluted 100 times with water. The second PCR was performed using 0.25 μl of the diluted first PCR products as the template and a pair of nested primers, β_A-U-3 (5'-GGCGGTCAAGAGGCACAT-3')

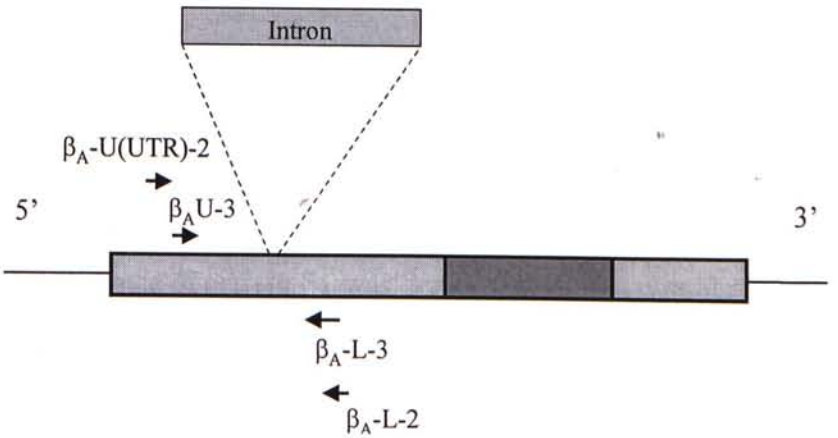


Fig. 2-2 Nested RT-PCR strategy. To avoid genomic DNA contamination, two pairs or primers (indicated by arrows) were designed to span a possible intron site according to the genomic structure of human activin β_A subunit (Tanimoto *et al.*, 1991). The hatched area represents the previously cloned genomic fragment.

and β_A-L-3 (5'-TGTCCACGGTCTTCTCGGA-3'), as the upper and lower primers, respectively. Both PCRs were carried out on Peltier Thermal Cycler 100 (MJ Research). Thirty cycles of reaction were performed using a profile of 94°C for 40 sec, 62°C for 30 sec and 72°C for 1.5 min after a 4-min-denaturation at 94°C and followed by a final 5-min-extension at 72°C. The PCR products were examined by electrophoresis in 1 % agarose gel.

2.3 Results

2.3.1 Cloning and sequence analysis of activin β_A cDNA

Since the presence of activin β_A was demonstrated in a number of goldfish tissues by immunocytochemistry (Ge *et al.*, 1993a), we screened different goldfish cDNA libraries from the ovary, pituitary and brain/pituitary for activin β_A by PCR using the primers designed according to the activin β_A fragment that we isolated previously (Ge *et al.*, 1993b). A brain and pituitary cDNA library was found to give the strongest signal (Fig. 2-3). The 5' and 3' ends of the β_A cDNA were then amplified from this cDNA library using two rounds of PCR (Fig. 2-1). For the 5' end, a smear was obtained after the first PCR (Fig. 2-4). Part of this smear (size ranging from 900 to 1,900 bp) was purified and used as template for the nested PCR. For the 3' end, weak but distinct bands were obtained after the first PCR (Fig. 2-4). The PCR products were diluted 100 times and used as templates for the second round of PCR using nested primers. After the nested PCR, several strong, distinct bands were obtained (Fig. 2-4). A 5' fragment of about 1,800 bp and a 3' fragment of about 700 bp were excised and purified, and their identity confirmed by PCR using β_A-U-2 and β_A-L as primers (Fig. 2-5). To further verify the identity of the fragments obtained, the 5'and 3' ends of the 5' and 3' fragments, respectively, were sequenced.

Fig. 2-3 PCR screening on several goldfish cDNA libraries for activin β_A. 1, fully-grown ovary from HCG-injected fish; 2, fully-grown ovary I; 3, full-grown ovary II; 4, early vitellogenic ovary; 5, pituitary; 6, brain/pituitary; +, positive control; –, negative control.

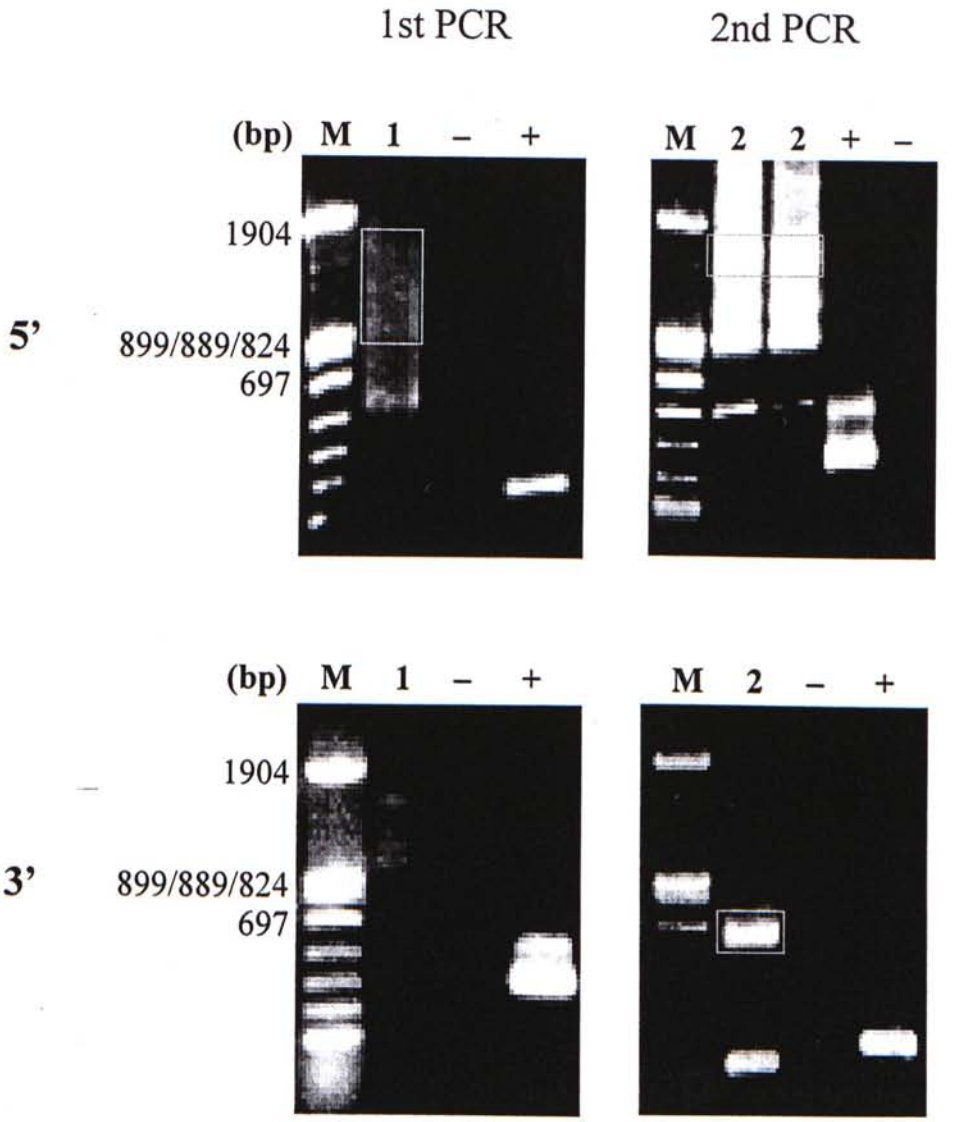


Fig. 2-4 Nested PCR. *Upper panels*: 5' direction. *Lower panels*: 3' direction. *Left panels*: 1st PCR. *Right panels*: 2nd PCR. Fragments to be excised were boxed (refer to text for details). M, size marker; 1, 1st PCR products; 2, 2nd PCR products; –, negative control; +, positive control.

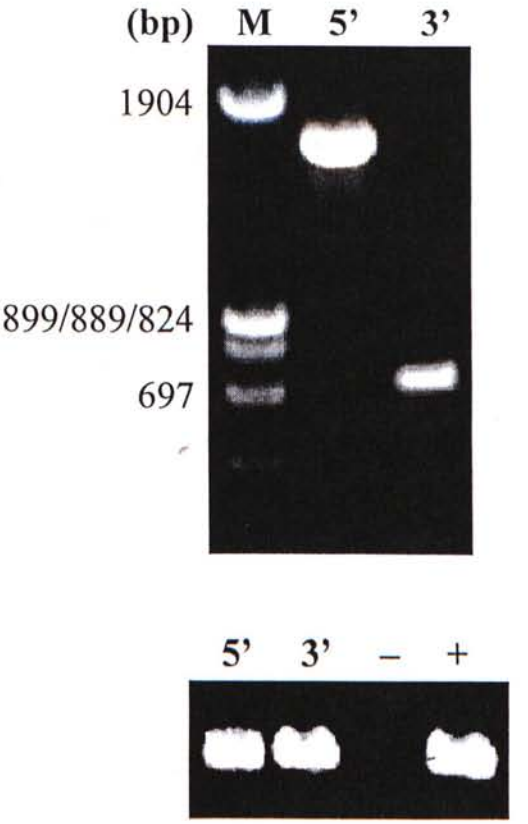


Fig. 2-5 Purified 5' and 3' fragments obtained after nested PCR. *Upper panel*: Purified 5' (~1,800 bp) and 3' (~700 bp) fragments. *Lower panel*: Confirmation of β_A identity using PCR. M, size marker; 5', 5' fragment; 3', 3' fragment; –, negative control; +, positive control.

GenBank search shows that the deduced amino acid sequences from both the 5' and 3' fragments share the highest homology with activin β_A subunit of other vertebrates (Table 2-1). These fragments were then linked by extension as described in Fig. 2-1 to obtain the putative full-length cDNA (Fig. 2-6). Both strands of the cDNA were sequenced on a series of overlapping subclones generated by exonuclease III and mung bean nuclease deletion (Fig. 2-7, 2-8, 2-9).

Open reading frame analysis revealed that the 1,792 bp cDNA clone encodes a protein of 404 amino acids (Fig. 2-10). GenBank search shows that the deduced amino acid sequence has the highest homology with the precursor of activin β_A subunit of other vertebrates (Table 2-2). The N-terminal of the precursor is a hydrophobic signal peptide with a potential cleavage site between position 23 and 24, as predicted by the computer program PLOT.A/SIG (Fig. 2-10, 2-11) using the method of von Heijne (1986). This agrees with the fact that activin A is a secreted protein. The mature peptide is situated at the C-terminal of the precursor. A potential cleavage site with 2 basic amino acids (KR) is identified at position 289. This suggests a mature peptide of 116 amino acids. A potential glycosylation site (NVT) is identified at position 80. No glycosylation site is found in the mature region, suggesting that mature activin A is not a glycoprotein. A putative polyadenlylation signal (AATAAA) is recognized 43 bp upstream from the poly(A) tail (Fig. 2-10). The predicted amino acid sequence of the isolated goldfish activin β_A cDNA shows 100% identity with the previously obtained genomic clone (Gact 18) encoding the mature peptide (Ge *et al.*, 1993b), suggesting that both of these clones code for the same functional protein even though the identity at the nucleotide level is only about 70% (Fig. 2-12). The nucleotide differences mainly occur at the

Table 2-1 BLASTN results for the 5' and 3' fragments.

5' fragment

Sequences producing High –scoring segment Pairs:		High Score	Smallest Sum Probability P(N)	N
Emb|X69619|MMBETA	*Mus musculus* mRNA for inhibin beta-A subunit	199	6.5e-06	1
gb|M37482|RATINHB	*Rattus norvergicus* inhibin beta-A-subunit mRNA, complete cds	199	6.5e-06	1
Emb|X57578XHSACTVBA1	*Homo sapiens* activin beta-A subunit (exon 1)	191	3.0e-05	1
gb|L19218|SHPBAINHBA	*Ovis aries* inhibin beta-A-subunit mRNA, complete cds	191	3.0e-05	1
gb|M13436|HUMINHBA	*H. sapiens* ovarian beta-A inhibin mRNA, complete cds	191	3.0e-05	1

3' fragment

Sequences producing High –scoring segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gb|U42377|GGU42377	*Gallus gallus* inhibin/activin beta A-subunit mRNA, complete cds	239	1.5e-09	1
gb|U26946|GGU26946	*G. gallus* inhibin beta A subunit mRNA, complete cds.	239	1.5e-09	1
gb|I01837	Sequence 4 from Patent US 4798885	228	1.3e-08	1
emb|X03266|SSINHBAR	*Sus scrofa* mRNA for inhibin beta (a)-subunit	228	1.3e-08	1
ab|I05233	Sequence 15 from Patent EP 0222491	228	1.3e-08	1

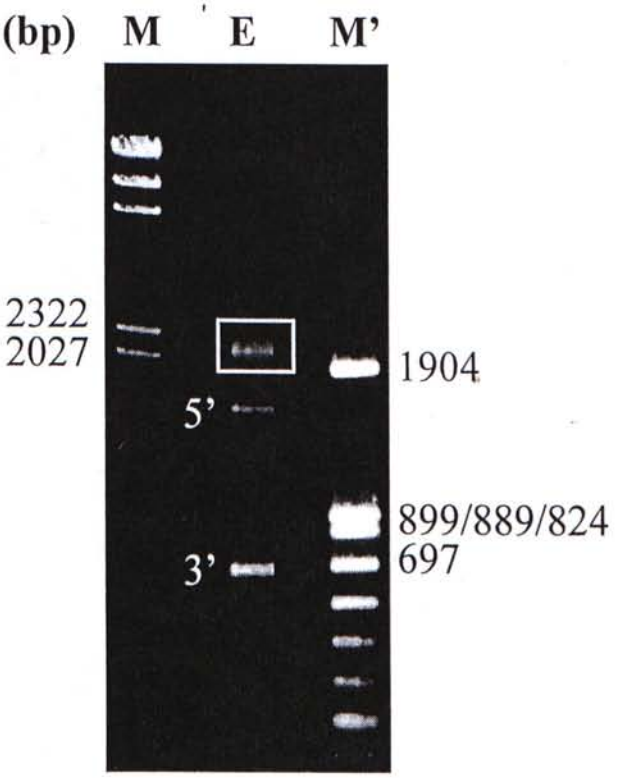


Fig. 2-6 Extension products containing the putative full-length activin β_A cDNA after the 15 cycles of extension. To minimize errors introduced by PCR, the number of cycles were kept at 15. The putative full-length cDNA is boxed. Note that the labelled 5' and 3' fragments, which served as both templates and primers, were not exhausted. M, M', size markers and E, extension products.

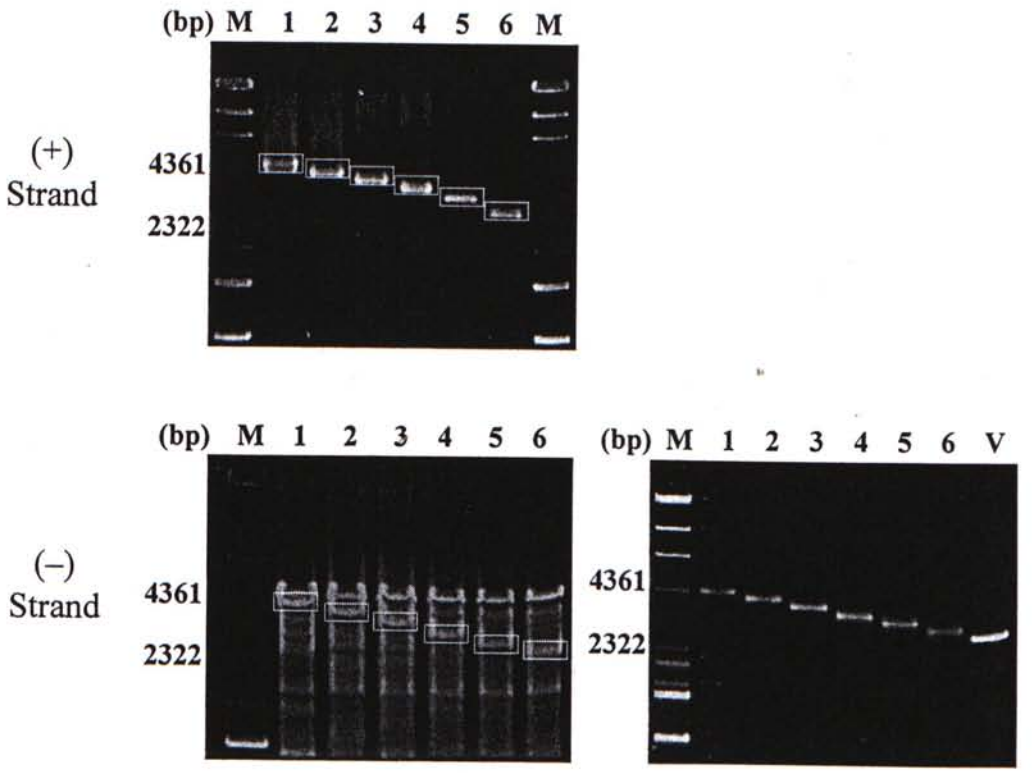


Fig. 2-7 Deletion fragments after exonuclease III and mung bean nuclease treatment. *Upper panel*: sense (+) strand. *Lower panels*: antisense (–) strand. Fragments to be excised are boxed. As a single sharp band was not obtained in the deletion mixture for the (–) strand, the purified fragments were shown on the right. M, size marker; 1-6, time points 1-6 at 1.5 min interval during the deletion reaction; V, vector alone without insert.

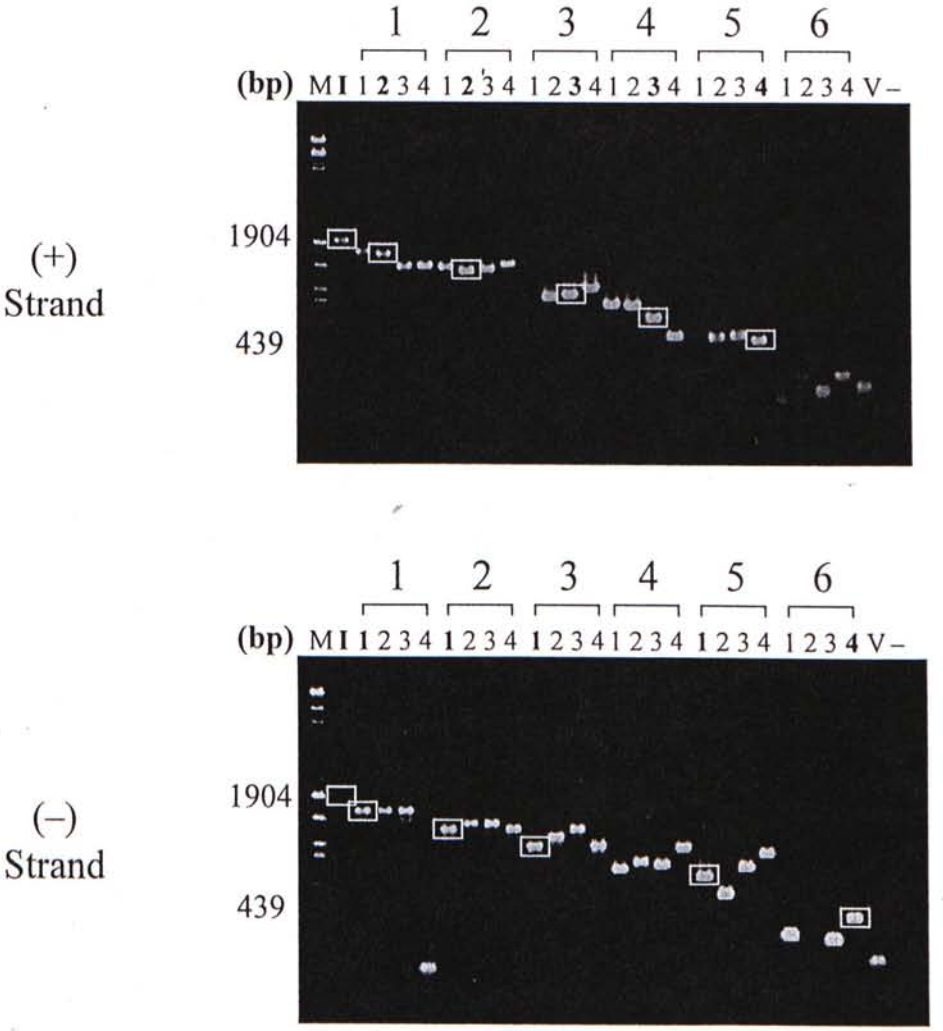


Fig. 2-8 PCR screening for overlapping subclones. *Upper panel*: sense (+) strand. *Lower panel*: antisense (–) strand. The subclones selected for sequencing are boxed. 1-6 (large font), time points 1-6 at 1.5 min interval during the deletion reaction; 1-4 (small font), subclones screened for each time point; M, size marker, I, intact plasmid with full-length insert; V, vector alone without insert; –, negative control. Note that even within the same time point, subclones of different sizes were generated.

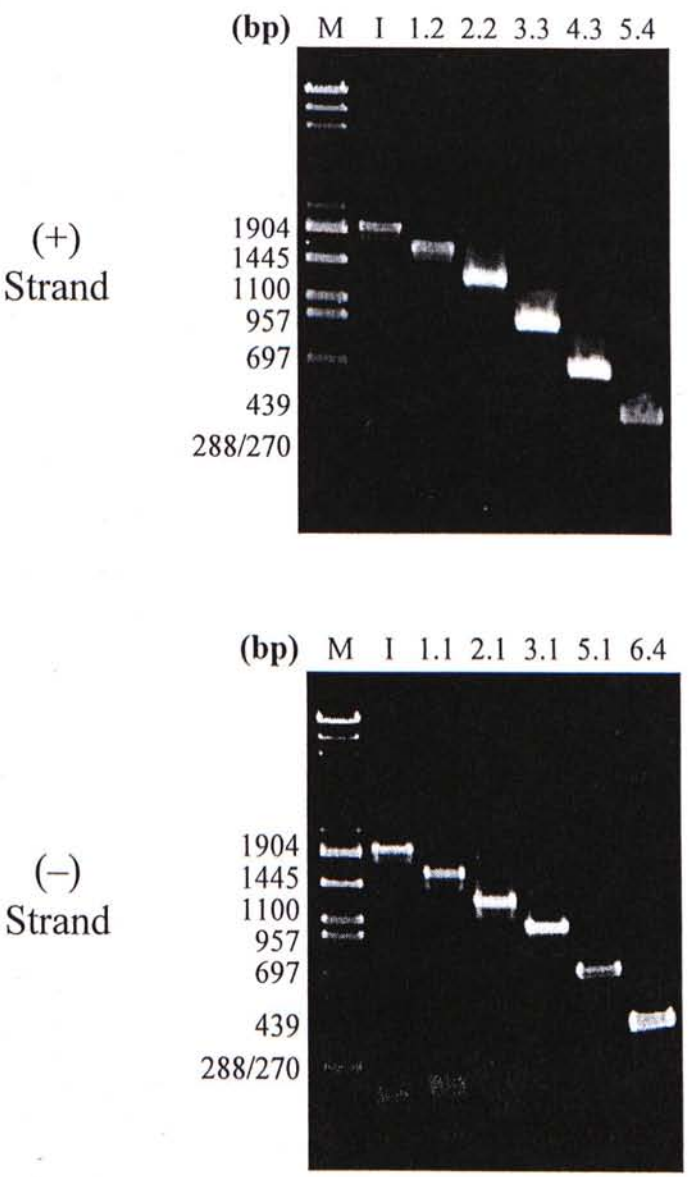


Fig. 2-9 Amplified products of the selected overlapping subclones for sequencing. *Upper panel*: sense (+) strand. *Lower panel*: antisense (–) strand. M, size marker; I, intact plasmid with full-length insert; 1.2, 2.2, 3.3, 4.3, 5.4 and 1.1, 2.1, 3.1, 5.1, 6.4, overlapping subclones for the (+) and (–) strands, respectively.

```
GGCACGGGCAGCCGTGCACCGTCAGGTTTCTCCAGAGCGACCCGCAGCTCATCGACCTGC      60
AGGAGGAGCTCACCGACGGCGCTTCGTTCAACCCTCTTCAACTTTTATCACCATGACCGC     120
TCCGCTCAGATAAGGATTATTTTGGACTGGCTCCCGCTTTCCGCATCGATTTGACAAGCA     180
AGCCAGCGCGGCGACTGTGATCTTGGCAGCGGAGCAGAACAGGACCGAGGTGTGAGTCTG     240
CCTTCACTCAGGATGTCCTCACTCACGCTCGTGAACAGGGGTACTGCTGCTCTCCGGCTG     300
            M  S  S  L  T  L  V  N  R  G  T  A  A  L  R  L
TTTGTCAGGGGGCTGCTCACCCACTCCAGCCGAGAGTGGCTCTCAGGGGACGGGGAGCCG     360
F  V  R  G  L  L  T  H  S  S  R  E  W  L  S  G  D  G  E  P
GATGATCCCGTGACCCCCTGCCCATCCTGCGCTCTAGCCCAAAGGCAGAAGGACTCAGAG     420
D  D  P  V  T  P  C  P  S  C  A  L  A  Q  R  Q  K  D  S  E
GAGCAGACCGACATGGTGGAGGCGGTCAAGAGGCACATCCTCAACATGCTGCATCTGAAC     480
E  Q  T  D  M  V  E  A  V  K  R  H  I  L  N  M  L  H  L  N
ACCCGGCCCAATGTCACACACCCGGTGCCCCGAGCCGCCCTGCTCAACGCCATCCGCAGG     540
T  R  P  N  V  T  H  P  V  P  R  A  A  L  L  N  A  I  R  R
CTGCACGTGGGACGGGTGGGGGAGGACGGCACAGTGGAGATGGAGGAAGATGGAGGGGGG     600
L  H  V  G  R  V  G  E  D  G  T  V  E  M  E  E  D  G  G  G
CTGGGGGAACACCGAGAGCAATCTGAGGAGCAGCCCTTCGAGATCATCACCTTCGCTGAG     660
L  G  E  H  R  E  Q  S  E  E  Q  P  F  E  I  I  T  F  A  E
CCAGGAGATGCCCCTGACATCATGAAATTTGACATTTCTATGGAGGGCAACACTCTGTCC     720
P  G  D  A  P  D  I  M  K  F  D  I  S  M  E  G  N  T  L  S
GTGGTGGAGCAGGCCAATGTGTGGCTGCTTCTTAAAGTTGCCAAGGGCAGCGGAGGAAAG     780
V  V  E  Q  A  N  V  W  L  L  L  K  V  A  K  G  S  R  G  K
GGGAAGGTGTCTGTGCAGCTCCTGCAGCATGGCAAAGCTGATCCAGGCTCTGCGGACGGA     840
G  K  V  S  V  Q  L  L  Q  H  G  K  A  D  P  G  S  A  D  G
CCCCAAGAAGCGGTGGTGTCCGAGAAGACCGTGGACACGCGGCGCAGTGGCTGGCACACA     900
P  Q  E  A  V  V  S  E  K  T  V  D  T  R  R  S  G  W  H  T
CTACCGGTGTCCCGCACAGTGCAGACCTTGCTGGACGGGGACAGCAGCATGCTTAGTCTC     960
L  P  V  S  R  T  V  Q  T  L  L  D  G  D  S  S  M  L  S  L
CGCGTCTCCTGTCCCATGTGTGCCGAAGCTGGCGCCGTGCCCATCCTGGTGCCCACAGAA    1020
R  V  S  C  P  M  C  A  E  A  G  A  V  P  I  L  V  P  T  E
AGCAACAAGGGTAAAGAGAGAGAACAATCTCACCGACCCTTCCTCATGGTCGTGCTCAAG    1080
S  N  K  G  K  E  R  E  Q  S  H  R  P  F  L  M  V  V  L  K
CCAGCCGAGGAGCACCCGCACCGGCGCAGCAAGCGCGGCCTGGAATGCGACGGCAAAATC    1140
P  A  E  E  H  P  H  R  R  S  K  R  G  L  E  C  D  G  K  I
CGTGTCTGCTGTAAACGGCAGTTCTACGTCAACTTTAAGGACATCGGGTGGAGCGACTGG    1200
R  V  C  C  K  R  Q  F  Y  V  N  F  K  D  I  G  W  S  D  W
ATCATCGCTCCGTCTGGGTATCACGCTAACTACTGCGAGGGCGACTGCCCCAGCCATGTG    1260
I  I  A  P  S  G  Y  H  A  N  Y  C  E  G  D  C  P  S  H  V
GCCAGCATCACAGGCTCTGCCCTCTCCTTCCATTCCACCGTCATCAATCACTATCGGATG    1320
A  S  I  T  G  S  A  L  S  F  H  S  T  V  I  N  H  Y  R  M
CGCGGGTACAGCCCTTTTAACAACATCAAGTCGTGCTGCGTACCTACCCGCCTACGGGCC    1380
R  G  Y  S  P  F  N  N  I  K  S  C  C  V  P  T  R  L  R  A
ATGTCCATGCTCTACTACAACGAGGAGCAGAAGATCATTAAGAAAGACATCCAGAACATG    1440
M  S  M  L  Y  Y  N  E  E  Q  K  I  I  K  K  D  I  Q  N  M
ATAGTGGAGGAGTGTGGCTGCTCATAAGACGCCATGAACTCCTAACGAACACTTCAATGT    1500
I  V  E  E  C  G  C  S  *
TGGTACAAATCACATATCAGAGGATCGGCAGTCTGTGTTCTCCCACAAACGTTTGAACTG    1560
CAAACTCGGGCAGCCGGTTTGTTTCTAGATCATTCCAAAAGGACTGATTGTTGGAGTGGT    1620
CTTTGTGGCACTTTCATATAAATATGACGGAGGAGAATCAAGCATAATATATTTTTTTTA    1680
ATGAAGGGAGGCTGAGAGGGAACGGGTGCATGAGCCCTCATTCTCTGCACTGC AATAAAG    1740
AGATTTATTTTGAAGTGTGGAATTATACATTCTGAGGAAAGAAAAAAAAAAA            1792
```

Fig. 2-10 DNA and deduced amino acid sequences of goldfish activin β_A subunit (Gactβ_A) (Yam *et al.*, 1999b; GenBank accession number AF169032). The signal peptide is underlined and the C-terminal mature region double-underlined. The potential glycosylation site (NVT) is marked by dotted underline. The two basic amino acids (KR) signifying the potential cleavage site and the polyadenylation signal (AATAAA) are in bold.

Table 2-2 BLASTX results for the putative full-length goldfish activin β_A cDNA.

Sequences producing High –scoring segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
sp|P43032|INBA_SHEEP	INHIBIN BETA A CHAIN PRECURSOR (ACTIVIN BETA-A CHAIN) pir||I47072 inhibin beta-A chain precursor – sheep gi|310380 (L19218) inhibin beta-A-subunit [*O. aries*]	+2	670	9.8e-127	6
sp|P07995|INBA_BOVIN	INHIBIN BETA A CHAIN PRECURSOR (ACTIVIN BETA-A CHAIN) pir||S50898 inhibin beta-A chain precursor – bovine gi|563749 (U16239) betaA inhibin/activin precursor [*Bos taurus*]	+2	666	4.7e-126	6
sp|P08476|INBA_HUMAN	INHIBIN BETA A CHAIN PRECURSOR (ACTIVIN BETA-A CHAIN) (ERYTHROID DIFFERENTIATION PROTEIN) (EDF) pir||B24248 inhibin beta-A chain precursor – human gi| 181947 (J03634) erythroid differentiation protein precursor [*H. sapiens*] gi|307069 (M13436) beta-A inhibin [*H. sapiens*] gi|825621 (X57578) activin beta-A subunit [*H. sapiens*] prf||1608260B inhibin betaA [*H. sapiens*]	+2	666	6.4e-126	6
sp|P55102|INBA_HORSE	INHIBIN BETA A CHAIN PRECURSOR (ACTIVIN BETA-A CHAIN) gnl|PID|d1009495 (D50326) inhibin beta A subunit [*Equus caballus*]	+2	652	4.1e-125	6
pir||JC4862	Activin beta-A chain – newt gnl|PID|d1013374 (D84516) activin beta-A subunit [*Cynops pyrrhogaster*]	+2	642	1.0e-121	6

The most likely cleavage site having the score 10.435

————————————|++ is between 23 I 24
———————————T|H+

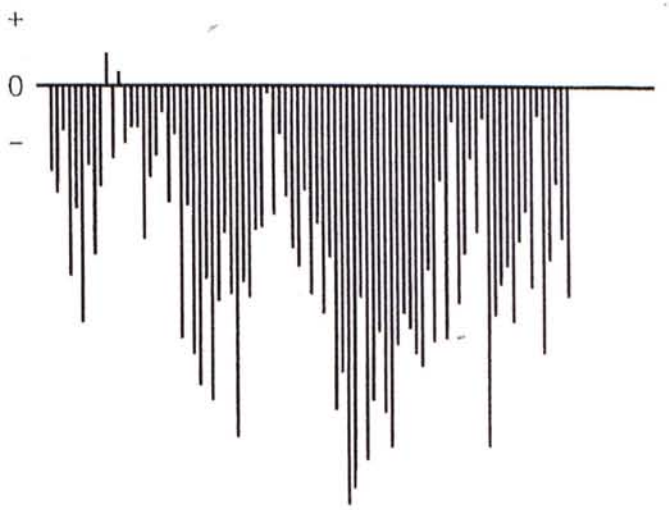


Fig. 2-11 Prediction of a hydrophobic signal peptide. A potential cleavage site between position 23 and 24 at the N-terminal of the activin β_A precursor is predicted by the computer program PLOT.A/SIG using the method of von Heijne (1986).

Fig. 2-12 A comparison of the nucleotide (*upper panel*) and predicted amino acid (*lower panel*) sequences of the mature peptide between goldfish activin β_A cDNA (Yam *et al.*, 1999b) and the genomic DNA fragment, Gact 18 (Ge *et al.*, 1993b).

third base of the codons and do not cause any change in amino acid in the mature protein. Goldfish activin β_A and β_B subunits share only 44% amino acid identity over the entire precursor and 60% over the C-terminal mature region (Ge *et al.*, 1993b, 1997a) (Fig. 2-13). The amino acid sequence identity between human and goldfish activin β_A subunit is 60% and 81% over the whole precursor and the mature region, respectively (Fig. 2-14). It is interesting to note a series of deletion in the goldfish activin β_A at position 258, which corresponds to a highly variable region in mammalian activin β_A. The nine cysteine residues in the mature region, which are important for the disulphide bond formation, are fully conserved among the species.

2.3.2 Distribution of activin β_A mRNA in different tissues

Activin has wide tissue distribution in mammals (Meunier *et al.*, 1988). The widespread distribution is also evident for goldfish activin β_B (Ge *et al.*, 1997a). To demonstrate if activin β_A is also diversely expressed, we examined its expression in different tissues using Northern blot analysis and RT-PCR. The specificity of the activin β_A probe was examined by Southern blot analysis. No crossreactivity with the β_B subunit was detected under the specified hybridization conditions (Fig. 2-15). Northern analysis revealed that activin β_A mRNA is expressed in the ovary, testis, brain and liver with transcripts of different sizes (Fig. 2-15). In the testis, two transcripts were detected with sizes being the same as that in the ovary and brain, respectively; however, the transcript of smaller size is much more abundant. No activin β_A mRNA was detected in the pituitary, even with the sensitive nested RT-PCR (Fig. 2-15).

```
beta-B    1:MDTL-F-KKMSIPI-LSVTCLMACILSVQCSLGAETVSQESQCASCGLGHPDDSGRMDTD
beta-A    1:.SS.TLVNRGTAALR.F.RG.LTHSSREWL.GDG.PDDPVTP.P..A.AQRQKDSEEQ..
            *  *           * *  *         *   *       * ** *          **

beta-B   61:FLEAVKRHILNRLQMRERPNITHPIPKAAMVTALRKLHAGKVREDGRVEI-PNFDGHAAH
beta-A   61:MV.........M.HLNT...V...V.R..LLN.I.R..V.R.G...T..MEEDGG.LGE.
          ********* *    *** *** * **   * * ** * * *** **      *   *

beta-B  121:NEVQEETS-EIISFAESDDVTPSKSSLYFLISNEGNQNLYVL-QANLWLYFKLLPGTQEK
beta-A  121:R.QS..QPF...T...PG.-A.--DIMK.D..M...-T.S.VE...V..LL.VAK.SRG.
          *  **   *** *** *  *      * ** ***  * *  *** **  *   *   *

beta-B  181:G-LRRKVTVRVRSYEPG-GQNVHWPMM-EKRVELKRSGWHTFPVSEAVREMLAKGGRRQD
beta-A  181:.KVSVQLLQHGKA-D..SADGPQEAVVS..T.DTR......L...RT.QTL.DGDSSMLS
          *              **          ** *   ****** ***  *   *

beta-B  241:LDIHCEGCEAANVLPILV--DP--S---DPSHRPFLVVRAQQADSK-HRIRKRGLECDGT
beta-A  241:.RVS.PM.AE.GAV....PTESNKGKEREQ......M.VLKP.EEHP..RS........-
          *   *  *  *   ****            ****** *    *    **  ********

beta-B  301:NGGLCCRQQFYIDFRLIGWNDWIIAPAGYYGNYCEGSCPAYMAGVPGSASSFHTAVVNQY
beta-A  301:KIRV..KR...VN.KD...S......S..HA.....D..SHV.SIT...L...ST.I.H.
          **  ***  *  *** ****** **  ***** **   *   *** ***  * * *

beta-B  361:RMRGISP-GSVNSCCIPTKLSTMSMLYFDDEYNIVKRDVPNMIVEECGCA
beta-A  361:....Y..FNNIK...V..R.RA.....YNE.QK.I.K.IQ.........S
          **** **     *** ** *  *****   *  * * *  *********
```

Fig. 2-13 A comparison between goldfish activin β_A (Yam *et al.*, 1999b) and β_B subunits (Ge *et al.*, 1997a). The C-terminal mature region is underlined.

Fig. 2-14 A comparison between goldfish activin β_A subunit (Yam *et al.*, 1999b) and those from human (Mason *et al.*, 1986), pig (Mason *et al.*, 1985), rat (Esch *et al.*, 1987) and mouse (Albano *et al.*, 1993). The C-terminal mature region is underlined and the conserved cysteine residues in the mature region are boxed.

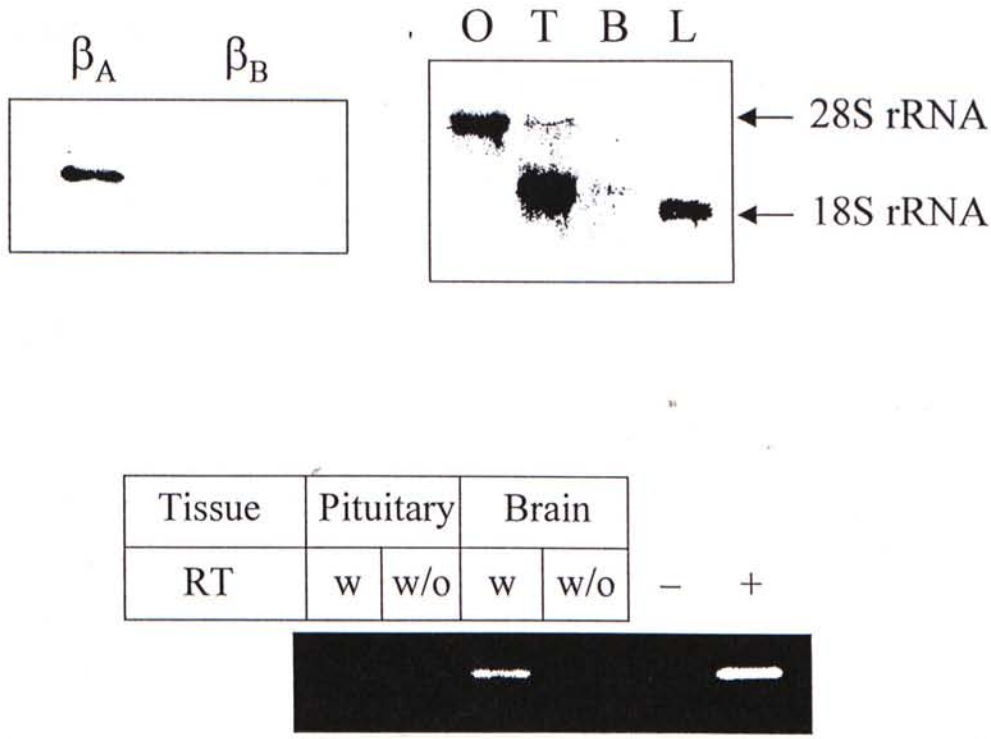


Fig. 2-15 Tissue distribution of activin β_A mRNA. *Upper left panel*: Southern blot analysis for the specificity of activin β_A probe. *Upper right panel*: Northern blot analysis showing the expression of activin β_A mRNA in different goldfish tissues. Seven, six, five and three micrograms of poly(A)$^+$ RNA were loaded for ovary (O), testis (T), brain (B) and liver (L), respectively. Positions of 28S and 18S rRNA are indicated on the right. *Lower panel:* RT-PCR analysis showing the expression of activin β_A mRNA in the goldfish brain and pituitary. (w, with RT; w/o, without RT; –, negative control with water as PCR template; +, positive control with activin β_A cDNA as PCR template)

2.4 Discussion

I have cloned a full-length cDNA of activin β_A (Gactβ_A) from a goldfish brain and pituitary cDNA library. The mature peptide, as deduced from the sequence analysis, shares 100% identity with that deduced from the genomic clone obtained previously (Ge *et al,* 1993b). This, together with our previous work (Ge *et al.*, 1993b), demonstrates the presence and expression of activin β_A subunit in the goldfish. Interestingly, though the predicted amino acid sequences of mature peptide between the two clones are identical, the identity at the nucleotide level is only about 70%. This strongly suggests the presence of different isoforms of the activin β_A subunit in the species. Recently, two short rainbow trout activin β_A genomic fragments were cloned (Tada *et al.,* 1998). Goldfish activin β_A shares about 90% amino acid sequence identity with the trout β_A over the mature region. Sequence analysis also shows that activin β_A is highly conserved across vertebrates. Over the mature peptide, goldfish activin β_A shares about 80% amino acid sequence identity with those of other vertebrates including humans. This high degree of conservation strongly argues for important roles played by activin in vertebrate life. However, when compared with activin β_B subunit, which shows an even higher amino acid sequence identity (93-95%) with those of mammals, activin β_A subunit appears to be more variable, suggesting functional difference between the two subunits. Since activin A and B have been reported to be equipotent in stimulating FSH secretion (Corrigan *et al.,* 1991) and erythroid differentiation (Mason *et al.*, 1989) in mammals, the question of why vertebrates produce two activin subunits with different degree of conservation during evolution remains to be answered. Comparative studies using different animal models, including fish, may shed light on

this issue.

Activin has wide tissue distribution in mammals (Meunier *et al.*, 1988). The widespread distribution is also evident for goldfish activin β_B (Ge *et al.*, 1997a, b). To demonstrate if activin β_A is also expressed in different tissues, we examined its expression at the mRNA level using Northern blot analysis. Similar to activin β_B, activin β_A mRNA is expressed in the ovary, testis, brain and liver (Fig. 2-15); however, unlike activin β_B that is expressed in the goldfish pituitary (Ge *et al.*, 1997a), no β_A mRNA signals were detected in the pituitary even with RT-PCR (Fig. 2-15). This appears to disagree with our previous study that showed the presence of immunoreactivities for both activin β_A and β_B subunits in the goldfish pituitary (Ge *et al.*, 1993a). This discrepancy between the two studies may be attributed to the presence and expression of different activin β_A isoforms. This is supported by the present finding that the β_A cDNA clone in this study shares only about 70% nucleotide sequence identity with the genomic clone encoding the mature peptide (Ge *et al.*, 1993b) despite the 100% amino acid sequence identity. This may explain why activin β_A could be detected in the immunocytochemical study, which was based on the amino acid sequence (Ge *et al.*, 1993a), but not in the present study using nucleotide-based detection methods (Northern blot analysis and RT-PCR). Moreover, Ge *et al.* (1993b) have also suggested the presence of activin β_A and β_B isoforms in the goldfish based on the presence of two bands in the Southern blot analysis of the genomic DNA. Ge *et al.* (1993b) have isolated two isoforms of activin β_B subunit but only one form of the β_A subunit. The present finding provides evidence for Ge *et al.* hypothesis. It is interesting to note that the two isoforms of

activin β_A are more variable (~70% nucleotide sequence identity in the mature peptide) than those of the β_B subunit, which share 95% identity (Ge *et al.*, 1993b). In the goldfish, the polymorphism of hormones and growth factors including prolactin (Chan *et al.*, 1996), growth hormone (Law *et al.*, 1996) and activin β_B subunit (Ge *et al.*, 1993b) has been well documented. This kind of polymorphism that is commonly observed in tetraploid teleosts appears to arise from the duplication of their genome as believed by Melamed *et al.* (1998). The present finding strongly suggests that goldfish activin β_A subunit is also polymorphic.

The observation that activin β_A is expressed in the brain but not the pituitary suggests that the full-length cDNA obtained from the brain and pituitary cDNA library is probably of brain origin and the pituitary is likely to express another isoform of activin β_A according to the above contention. The presence of activin in the brain has also been observed in the rat, in which it stimulates oxytocin release (Sawchenko *et al.*, 1988). However, the function of activin in the goldfish brain remains to be elucidated.

In mammals, the presence of activin β_A in the pituitary is controversial. Both forms of the β subunits were detected in porcine anterior pituitary cells (Li *et al.*, 1997) and in a pituitary cDNA library from humans (Alexander *et al.*, 1995). In the rat, in which activin has been extensively studied, Meunier *et al.* (1988) and Roberts *et al.* (1989) showed that only the β_B but not the β_A mRNA was expressed in the pituitary. However, recent studies demonstrated that both activin β_A and β_B subunits were expressed in the rat pituitary when assayed by RT-PCR (Halvorson *et al.*,

1994) and in cultured pituitary cells by immunostaining (Liu *et al.*, 1996). It is interesting that all these studies consistently reported the expression of activin β_B subunit in the pituitary. Liu *et al.* (1996) suggested that activin A and B may be differentially regulated in the pituitary and play different roles, and the apparent discrepancies regarding β_A expression in the pituitary may be due to the different states of the pituitary used in different studies.

The presence of activin subunits in fish (Ge *et al.*, 1997a; Nagahama, 1994) and mammalian testes (Vliegen *et al.*, 1993) suggests that the involvement of activin in the regulation of male reproduction is well conserved across vertebrates. Activin β_A is abundantly expressed in the human testis (Vliegen *et al.*, 1993) and activin A stimulates DNA synthesis during rat spermatogenesis (Hakovirta *et al.*, 1993). In fish, activin β_B subunit has been identified in the Japanese eel testis by subtractive hybridization and activin B was shown to play an important role in gonadotropin-induced spermatogenesis (Nagahama, 1994). The result that activin β_A is abundantly expressed in the goldfish testis from the present study suggests that activin A could also participate in the regulation of spermatogenesis in the goldfish. The strong expression of activin β_A in the goldfish ovary is consistent with the observation in the rat (Woodruff *et al.*, 1987) and chicken (Chen and Johnson, 1996a), which suggests that the ovary is the major production site of activin β_A. Interestingly, in contrast to the observation in other tissues (testis, brain and liver) where the expression levels of activin β_A were more or less similar between different samples, the level of β_A mRNA in the goldfish ovary varied tremendously from sample to sample, and sometimes no signals could be detected (data not shown). This

discrepancy may be due to the different stages of ovaries used in the experiments, which showed dramatic morphological and physiological changes throughout the reproductive cycle. The significant variation of activin β_A expression in the ovary suggests that activin β_A is tightly controlled and is likely to play important roles in fish ovarian functions. This, if confirmed, would be in agreement with the situation in the chicken in which activin β_A mRNA level changes dramatically with the development of the preovulatory follicles (Chen and Johnson, 1996b). Similar to the β_B, activin β_A mRNA is also expressed in the goldfish liver, but at a surprisingly high level. This finding, together with our observation that activin receptors are also expressed in the goldfish liver (Ge *et al.,* unpublished), suggests that activin may act as an autocrine/ paracrine factor to regulate liver functions. The high level of expression of activin β_A has also been reported in the chicken liver (Chen and Johnson, 1996a).

Our Northern analysis showed that the four tissues (ovary, testis, brain and liver) express activin β_A mRNA transcripts of different sizes, a phenomenon also reported in the human (Dolter *et al.,* 1998), rat (Woodruff *et al.*, 1987) and chicken (Chen and Johnson, 1996a). This may be due to different transcription start sites (Feng *et al.,* 1995), alternative RNA splicing (Dolter *et al.,* 1998) and different polyadenylation sites (Tanimoto *et al.,* 1993).

In summary, we have cloned, for the first time in a teleost, a full-length cDNA encoding activin β_A subunit in the goldfish (Gactβ_A), and demonstrated that it is expressed in several tissues including ovary, testis, brain and liver, with transcripts

of different sizes. The isolated goldfish activin β_A, as a less conserved form of activin as compared to its β_B counterpart, may have an isoform with about 70% nucleotide identity in the mature peptide, and will provide a necessary tool for studies on the functional evolution of these physiologically and developmentally important molecules.

Chapter 3

Establishment and Characterization of Stable Cell Lines for the Recombinant Production of Goldfish Activin A

3.1 Introduction

In the last chapter, I described cloning of a putative cDNA in the goldfish (Gactβ_A) that shows the highest homology with mammalian activin β_A. In this chapter, I will confirm the functional identity of the clone using EDF (erythroid differentiation factor)-assay, a well-established bioassay specific for activin (Eto *et al.*, 1987; Yu *et al.*, 1987).

Since activin was identified, different methods have been adopted to purify it from natural sources (Ling *et al.*, 1985, 1986a, b; Vale *et al.*, 1986). However, as a growth factor, activin is not abundant in animal tissues and the yield of purification is generally low. Therefore, recombinant production has become the major source of activin for scientific research. Both recombinant activin A (Murata *et al.*, 1988; Schwall *et al.*, 1988) and B (Mason *et al.*, 1989; Schmelzer *et al.*, 1990) have been produced in mammals and served as valuable sources of activins for a vast array of studies contributing to our present knowledge of activin, especially in the mammalian species. To date, the studies in non-mammalian species including fish also rely on the use of the mammalian molecules (Ge *et al.*, 1992, 1994; Kagawa *et al.*, 1994). However, problems of specificity and efficacy may arise when mammalian activins are used in the non-mammalian models. To further study the

Part of this chapter was published in "Yam KM, Yu KL and Ge W (1999b) Cloning and characterization of goldfish activin β_A subunit. *Mol Cell Endocrinol* **154**:45-54".

physiological relevance of activin in fish, it would be desirable to have activin available from the same species origin.

Recently, goldfish activin B has been produced in our laboratory (Ge *et al.*, to be submitted). This serves as an important source of activin B for studies on its regulation of reproduction and development in the species. With the cDNA for goldfish activin β_A available, the present study also aims at establishing and characterizing stable cell lines that produce high level of recombinant goldfish activin A. This, together with the recombinant goldfish activin B currently produced in our laboratory, makes goldfish the only non-human model in which both recombinant activin A and B are available for detailed comparative studies on their functions.

3.2 Materials and Methods

All chemicals were obtained from Sigma (St. Louis, MO) and enzymes from Promega (Madison, WI) unless otherwise stated.

3.2.1 Construction of expression plasmid

The full-length goldfish activin β_A cDNA was separated from the plasmid pKS/Gactβ_A after digestion with *Eco*RI and *Xho*I. This was subcloned into the respective sites of the expression vector pBK-CMV (Stratagene) to generate the construct pBK/Gactβ_A (Fig. 3-1). The lac promoter of the vector was removed by digestion with *Nhe*I and *Bss*HII so that the insert is directly controlled by the CMV promoter. The plasmid contains a neomycin resistant gene, which allows for G418 selection.

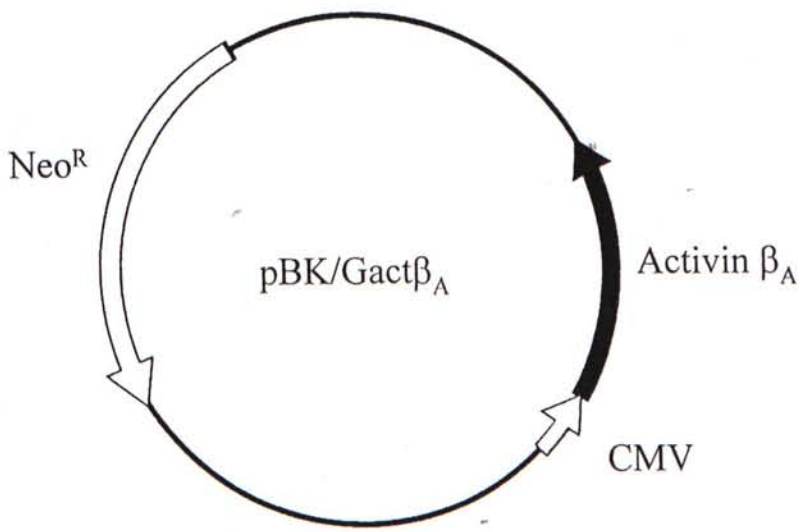


Fig. 3-1 The expression construct, pBK/Gactβ_A.

3.2.2 Cell Culture

Chinese hamster ovary (CHO) cells were cultured at 37°C with 5% CO_2 in MEM alpha (+) medium (Gibco BRL, Gaithersburg, MD) with 10% foetal bovine serum (Hyclone Laboratories, Logan, UT). F5-5 cells were cultured at 37°C with 5% CO_2 in Ham's F12 medium (Gibco BRL) with 10% foetal clone III serum (Hyclone).

3.2.3 Transfection of CHO cells

Twenty four hours before transfection, confluent CHO cells were subjected to a 1:8 subculture in 5 ml of the culture medium in 60 mm tissue culture plate. These were transfected with the construct pBK/Gactβ_A using the calcium phosphate-DNA coprecipitation method (Sambrook *et al.*, 1989). Briefly, thirty micrograms of the plasmid DNA in 360 µl of H_2O were mixed with 40 µl of 2.5 M $CaCl_2$ and 400 µl of 2X BES-buffered saline (50 mM BES, 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 2H_2O$). The mixture was incubated at room temperature for 20 min, and added dropwise to the cells. Twenty-four hours after transfection, the cells were refreshed with non-selective medium and incubated for another 24 hours before selection.

3.2.4 G418 selection of transfected CHO cells

Confluent transfected cells were subcultured at 1:4 dilution in non-selective medium for 24 hours. The cells were then cultured in selective medium with the optimal G418 concentration as determined by the test described below. After 1 week, when large colonies were formed, the cells were cultured in non-selective medium for 4 days before the medium was taken for EDF-assay. To determine the optimal G418 concentration for selection, confluent CHO cells were subcultured at 1:3 dilution in different concentrations (0-800 µg/ml active) of G418 sulphate

(Gibco BRL). The minimal concentration that kills all the cells was regarded optimal and used for selection.

3.2.5 Activin bioassay (EDF-assay)

The erythroid differentiation factor (EDF)-assay was used to detect activin activity of inducing F5-5 cell differentiation (Asashima *et al.*, 1991; *Eto et al.*, 1987) in the medium from transfected and cloned CHO cells according to Eto *et al.* (1987). The assay was carried out in 96-well plates using F5-5 cells, a mouse leukaemia cell line that specifically responds to activin by differentiating into haemoglobin-producing cells. Briefly, ten to forty microlitres of the culture medium from the transfected CHO cells were added to each well containing 3×10^3 F5-5 cells in a final volume of 200 μl. After incubation for 5 days at 37°C with 5% CO_2, the cells were fixed and stained for haemoglobin with *o*-dianisidine and H_2O_2. (Eto *et al.*, 1987). The positive cells (blue/dark brown) were counted and expressed as the percentage of the total (Fig. 3-2). Data were statistically analysed by Student's *t*-test. $P<0.05$ was considered statistically significant.

3.2.6 Cloning of pBK/Gactβ_A-transfected CHO cells by limited dilution

pBK/Gactβ_A-transfected CHO cells were subcultured in a 24-well plate at a density of 2-3 cells per well. The medium was collected for activin bioassay after a 3-day-incubation in non-selective medium following selection. Individual clones were obtained from the positive wells by limited dilution in 96-well plates.

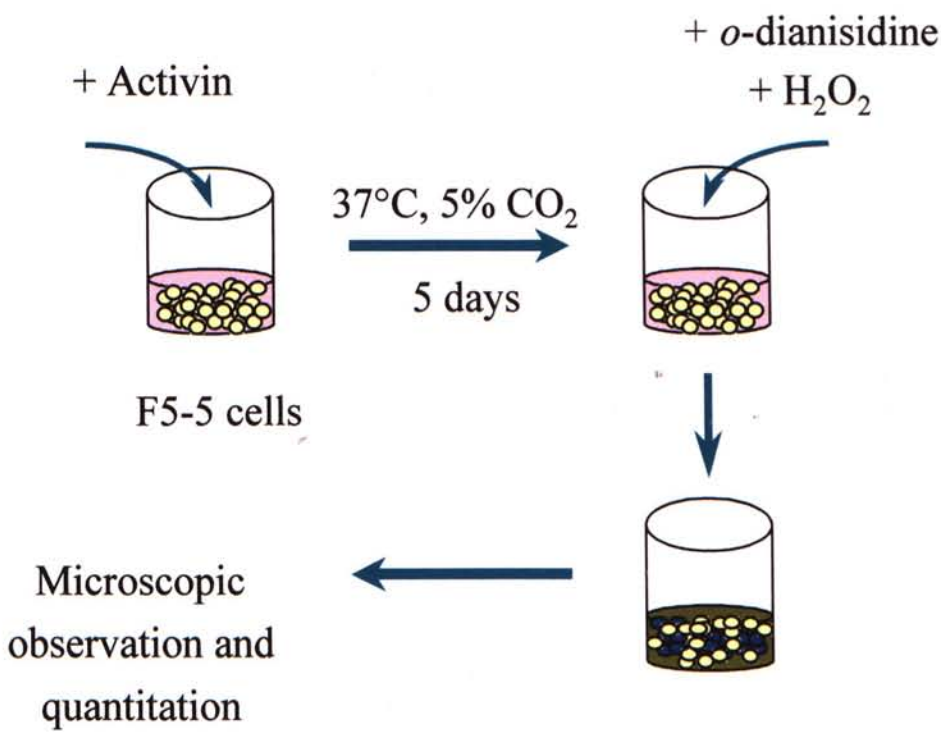


Fig. 3-2 EDF-assay. Culture medium that may contain activin was added to the F5-5 cells. After a 5-day-incubation, the cells were fixed and stained for haemoglobin with *o*-dianisidine and H_2O_2 (Eto *et al.*, 1987). The positive cells (blue/dark brown) as observed under microscope were counted and expressed as a percentage of the total.

3.2.7 Isolation of total RNA

Total RNA was isolated from the cultured CHO cells using TRI Reagent (Molecular Research Centre, Inc, Cincinnati, OH) according to the manufacturer's protocol. Briefly, cells were suspended by trypsinization, transferred to a 1.5 ml microtube and mixed with 1 ml of TRI Reagent. After a 5-min-incubation at room temperature, two hundred microlitres of chloroform was added and the mixture was incubated at room temperature for 10 minutes. The aqueous layer was separated from the organic layer by centrifugation, and transferred to a fresh microtube. Half a millilitre of isopropanol and 0.5 μl of glycogen (20 mg/ml) were added for precipitation. After a 10-min-incubation at room temperature, the total RNA was pelleted, washed with 75% ethanol and dissolved in formamide.

3.2.8 Northern blot analysis

Ten micrograms of total RNA from pBK/Gactβ_A-transfected CHO cell clones were electrophoresed in 1.1% denaturing gel [1.1% agarose in 1X MOPS (40 mM MOPS, 10 mM NaOAc and 1 mM EDTA, pH 7.0) and 2.2 M formaldehyde], blotted onto positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) using VacuGene XL System (Pharmacia, Uppsala, Sweden), and UV-crosslinked at 150 mJ using GS GeneLinker (Bio-Rad, Hercules, CA). A full-length Gactβ_A cRNA probe was labelled with digoxigenin (DIG) by *in vitro* transcription using 1 μg linearized DNA, 1X DIG-RNA Labelling Mix (Boehringer Mannheim), 40 U T_3 RNA polymerase and 10 mM DTT in 1X Transcription Buffer. The nylon blot was hybridized overnight with the probe (25 ng/ml) at 50°C in hybridization solution containing 5X SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent (Boehringer Mannheim) and 50% formamide after a 2-hour-

prehybridization at 50°C in the hybridization solution without the probe. After hybridization, the blots were washed twice at room temperature with 2X SSC/0.1% SDS for 5 min each and twice at 65°C with 0.5X SSC/0.1% SDS and once with 0.1X SSC/0.1% SDS for 15 min each. Detection was performed using the Chemiluminescence Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, the membrane was blocked with blocking solution [1:10 w/v Blocking Reagent/maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5)] at room temperature for 1 hour after being equilibrated in washing buffer [maleic acid buffer, pH 7.5 and 0.3% w/v Tween 20]. The blocked membrane was incubated with anti-DIG antibodies (1:10,000) at room temperature for 30 min, washed twice in washing buffer for 15 min each, and equilibrated in detection buffer (100 mM Tris-HCl and 100 mM NaCl, pH 9.5). Substrate solution (1:100 v/v CSPD/detection buffer) was applied to the membrane followed by exposure to the X-ray film.

3.3 Results

3.3.1 Optimization of G418 concentration for selection

G418 allows selection of stable clones that have the plasmid integrated into the genome and express the neomycin resistant gene (Neo^R). Optimal G418 concentration is essential for effective selection for maximal number of stable clones carrying the gene of interest. If the concentration is too low, some transient clones may also be selected; on the other hand, if the concentration is too high, the clones that express low level of Neo^R but high level of activin may be lost. To optimize the concentration of G418, confluent CHO cells were subjected to a 1:3 subculture.

These were then treated with different concentrations of G418 (0-800 μg/ml). As shown in Fig. 3-3, the number of colonies was reduced in the cultures containing 100-200 μg/ml G418 as compared to the control, and signs of cell death were evident. At a concentration of 400 μg/ml, most cells were dead and only a few small, unhealthy colonies were observed. When the concentration of G418 was increased to 500-800 μg/ml, no colonies could be seen, and only a few isolated, unhealthy cells were found. Therefore, 500 μg/ml was the minimal concentration that could kill all CHO cells without transfection and prevent colony formation, and was used as the optimal concentration for the selection of stable clones.

3.3.2 Expression of activin activity by pBK/Gactβ_A-transfected CHO cells

The functional identity of the Gactβ_A clone was confirmed using an EDF-assay after the cDNA had been subcloned into the expression vector pBK-CMV (pBK) and transfected into CHO cells followed by G418 selection (Fig. 3-4, 3-5). Specific activin activity of inducing the F5-5 cells to differentiate into erythroid cells was observed in the medium from the pBK/Gactβ_A-transfected cells (17±3% differentiation) and no activity was detected in the medium from the cells transfected with the vector alone (Fig. 3-6).

3.3.3 Establishment and characterization of CHO cell lines that stably produce recombinant goldfish activin A

The Gactβ_A-transfected cells with activin activity were subjected to two rounds of limited dilution (Fig. 3-7). After the first round of dilution, two wells (B4 and D1) that expressed high levels of activin activities were chosen. These were further

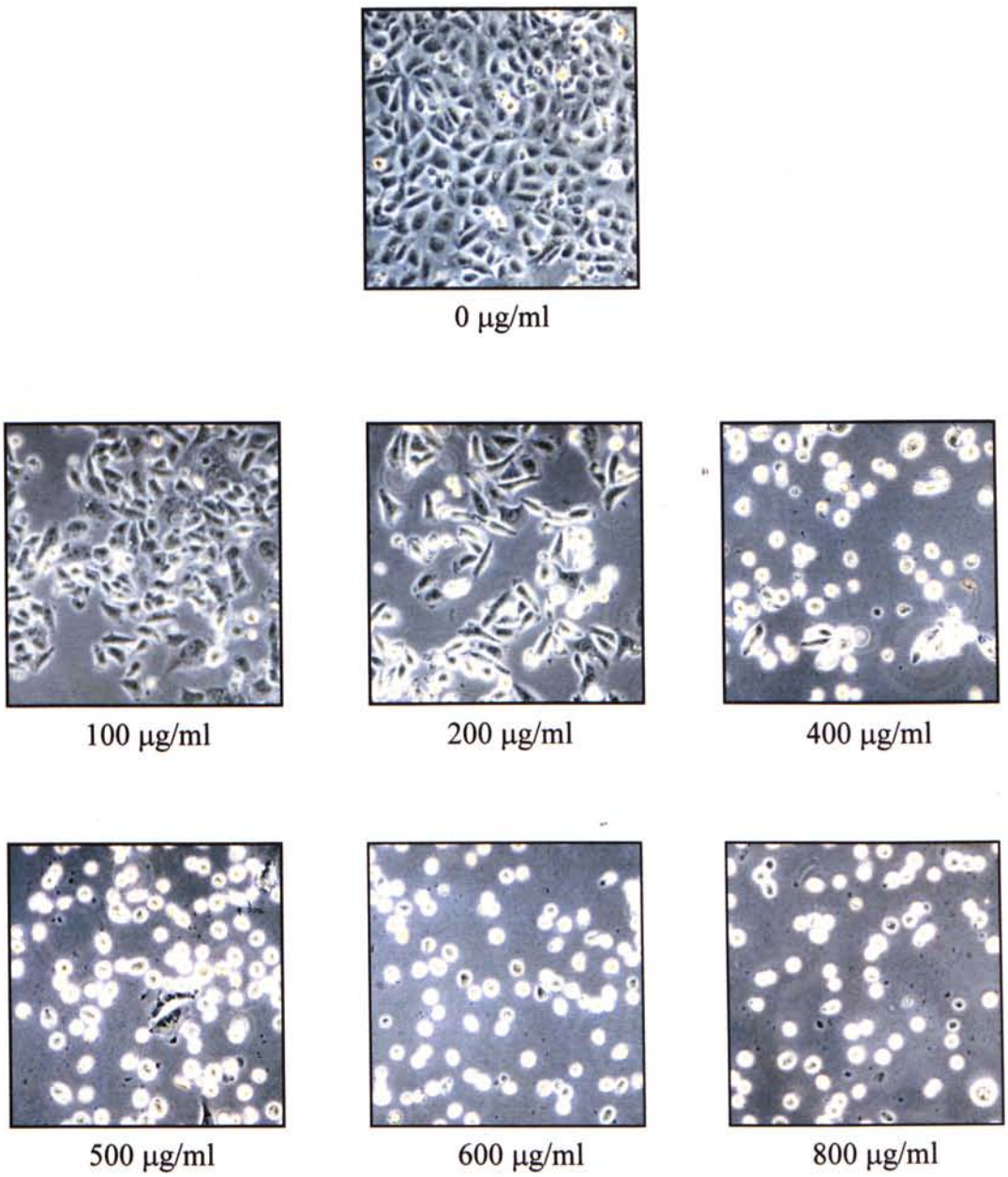
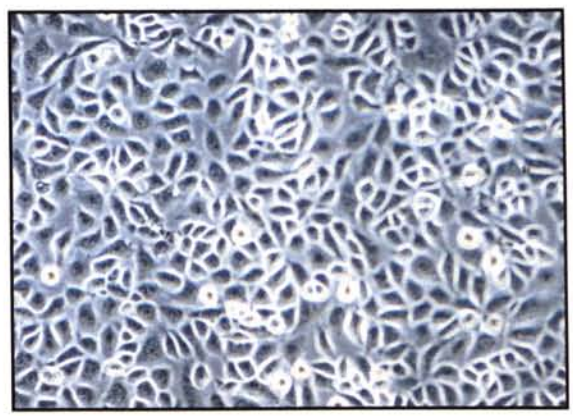
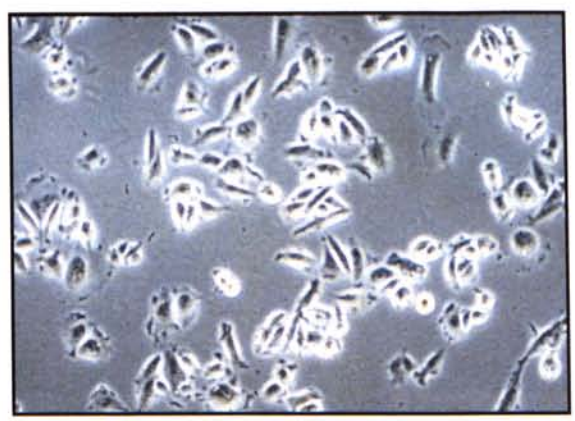


Fig. 3-3 Determination of the optimal G418 concentration for selection. CHO cells were treated with different concentrations of G418 (0-800 μg/ml). The minimal concentration that kills all the cells was regarded optimal and used for selection. Magnification, X400.

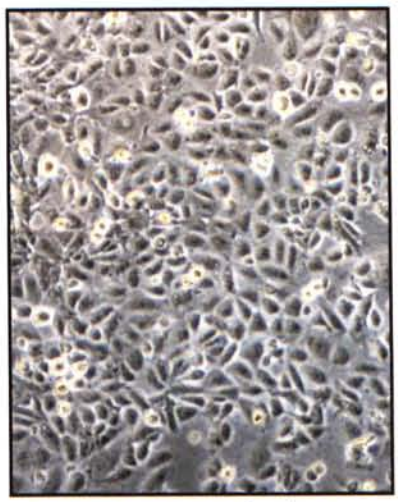


Confluent CHO cells



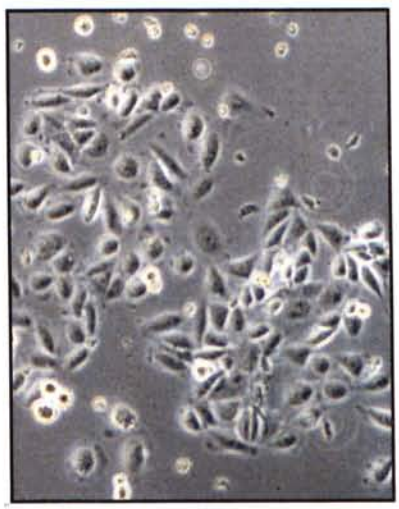
1:8 subcultured CHO cells right before transfection

Fig. 3-4 CHO cells before transfection. Magnification, X400.

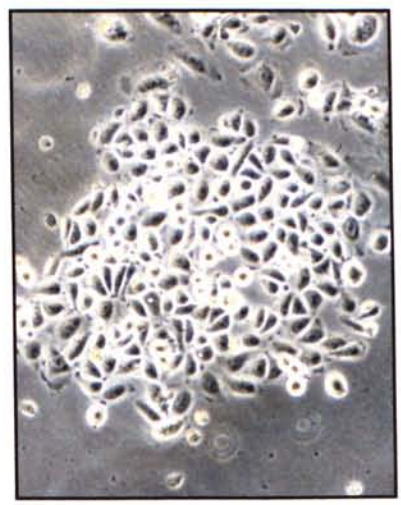


Control w/o transfection
and selection

Control w/o transfection
but under G418 selection



pBK-transfected cells
under G418 selection



pBK/Gactβ_A-transfected
cells under G418 selection

Fig. 3-5 Transfection and selection of CHO cells. Magnification, X400.

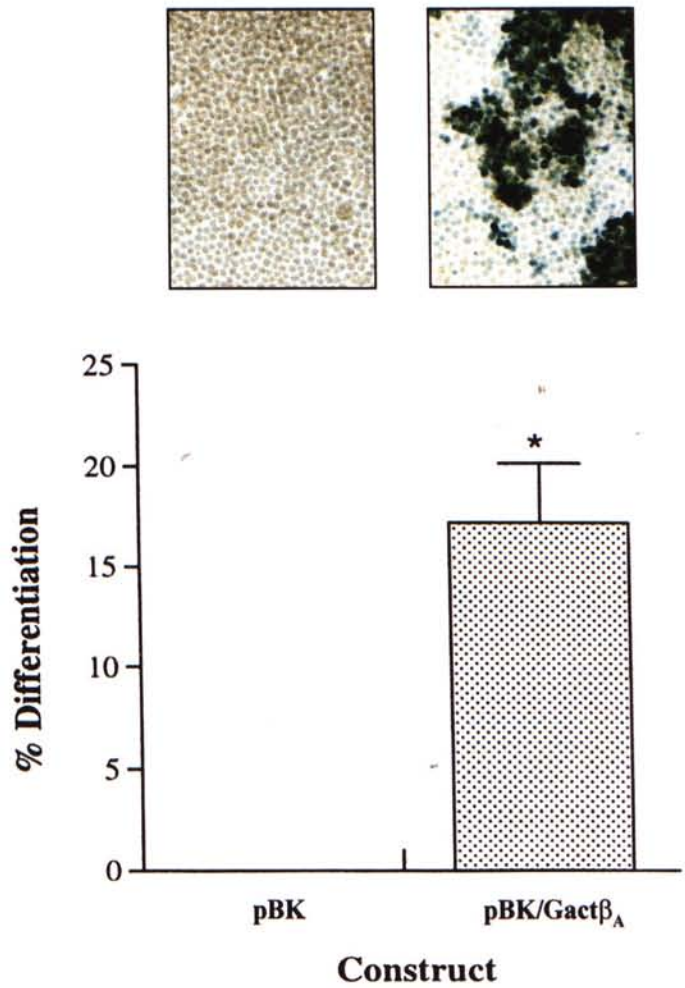


Fig. 3-6 Specific EDF-activity of activin in the conditioned medium from the Gactβ$_A$-transfected CHO cells. *Upper panel*: Haemoglobin detection in F5-5 cells after a 5-day-incubation in the presence of pBK- and pBK/Gactβ$_A$-transfected CHO cell medium. Magnification, X200. *Lower panel*: F5-5 differentiation induced by the conditioned medium. Data represent mean±SEM of three determinations. *$p<0.005$.

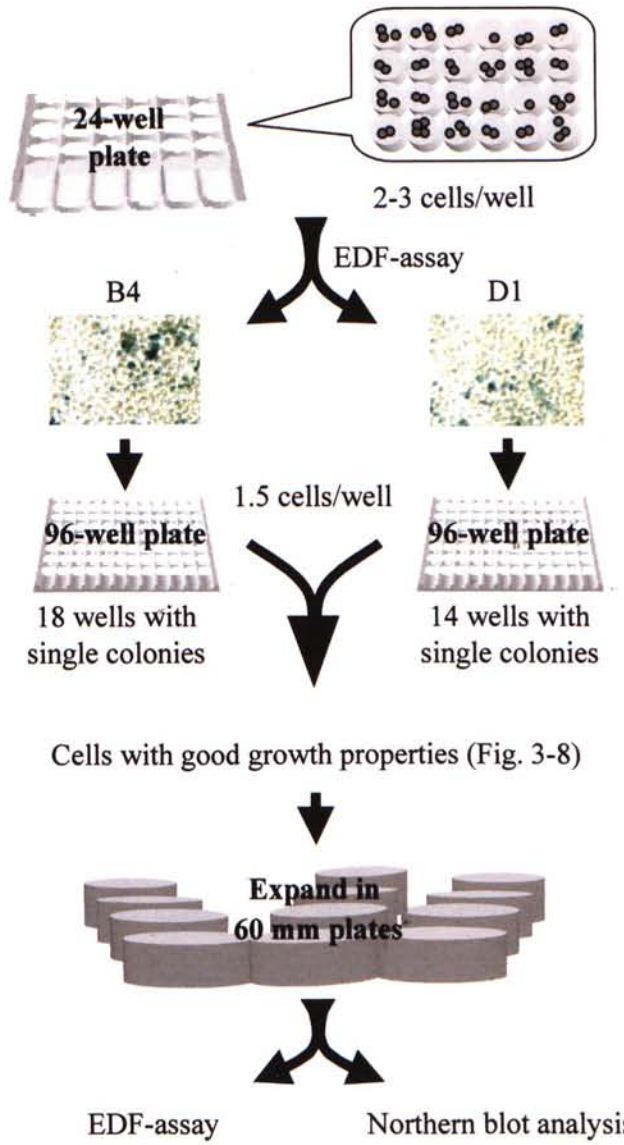


Fig. 3-7 Cloning of activin A-producing CHO cells by limited dilution. pBK/Gactβ_A-transfected CHO were subcultured in a 24-well plate at a density of 2-3 cells per well. The medium was collected for activin bioassay after a 3-day-incubation. Two wells (B4 and D1) showing high level of activin activity were chosen and further diluted in 96-well plates at a density of 1.5 cells per well. Wells showing high activin activity and with good growth properties were expanded for subsequent analyses.

diluted in 96-well plates at a density of 1.5 cells/ well. Single colonies were found in 18 wells from B4 and 14 wells from D1. Media from these wells were screened for activin activity with EDF-assay. Seven colonies with good growth properties were expanded by subculturing (Fig. 3-8). The conditioned medium from each clone was collected for EDF-assay when the culture reached confluency, and the total RNA extracted for subsequent Northern blot analysis. Three clones expressed activin β_A mRNA at different levels with D1C7 and D1G10 the highest (Fig. 3-9). The media from these three clones could induce significant F5-5 cell differentiation of different levels (15-30%) (Fig. 3-9, 3-10).

3.4 Discussion

The present study was conducted to confirm the functional identity of the Gactβ_A clone. Full-length Gactβ_A cDNA was subcloned into an expression vector, pBK-CMV, and transfected into CHO cells. Specific activin activity of inducing F5-5 cells to differentiate into erythroid cells was observed in the EDF-assay. These results not only confirm the functional identity of the clone, but also provide evidence that the EDF activity of activin is well conserved across vertebrates (Eto *et al.*, 1987; Yu *et al.*, 1987).

The expression system chosen provided a good means of producing bioactive recombinant goldfish activin. First, the expression vector, pBK-CMV, is commonly used for eukaryotic expression and selection. The expression is driven by the CMV immedidate early promoter and signals for termination of eukaryotic transcription and polyadenylation are provided by the SV40 poly(A) signal. The

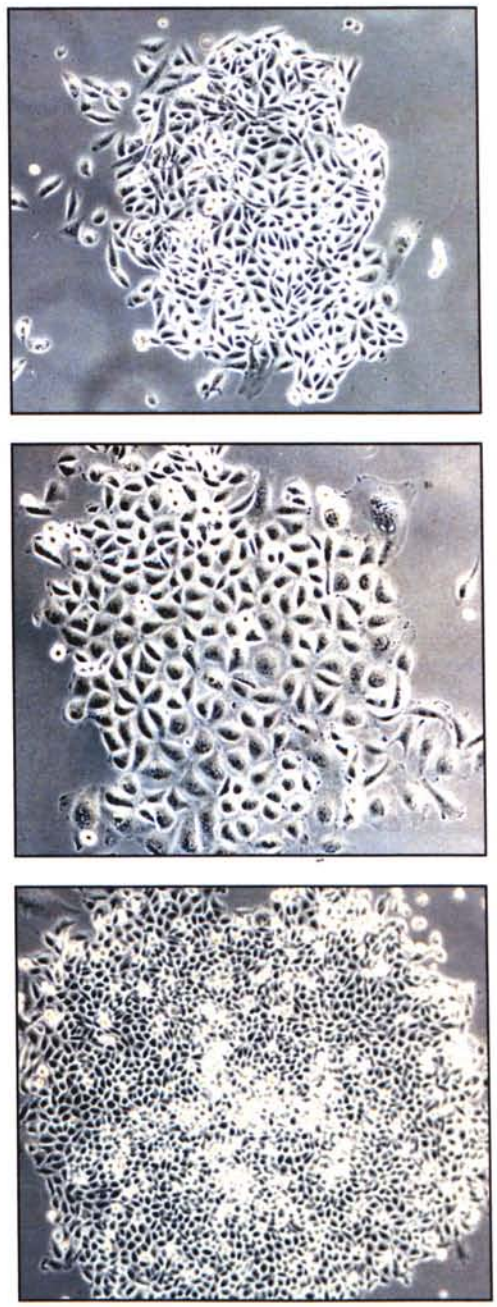


Fig. 3-8 Three of the healthy pBK/Gactβ_A-transfected CHO cell clones with different morphologies. Magnification, X200.

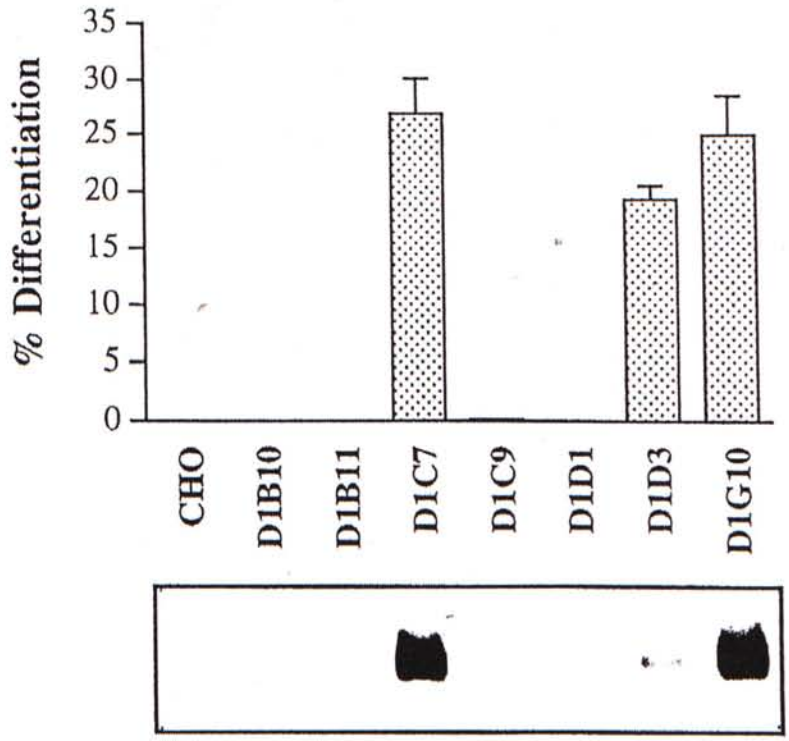
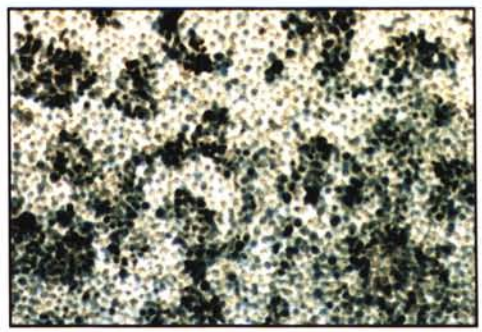
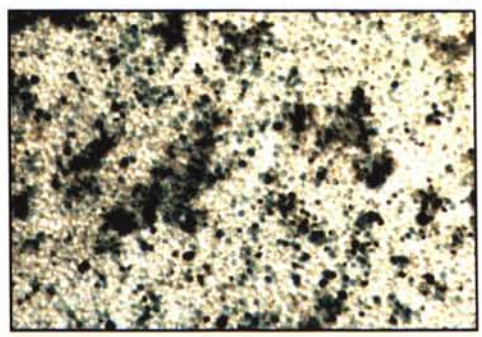


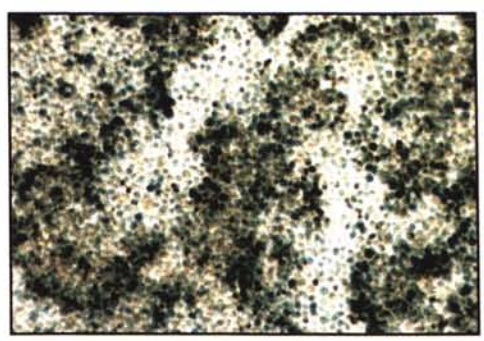
Fig. 3-9 Expression of activin A by cloned pBK/Gactβ_A-transfected CHO cells. *Upper panel*: EDF activity of activin in the conditioned media from transfected CHO cell clones. *Lower panel*: Northern blot analysis for activin β_A mRNA expressed by different CHO cell clones (D1B10, D1B11, D1C7, D1C9, D1D1, D1D3 and D1G10). Data represent mean±SEM of three determinations. CHO, control cells transfected with vector pBK.



D1C7



D1D3



D1G10

Fig. 3-10 Specific EDF-activity by the individual CHO cell clones (D1C7, D1D3 and D1G10). F5-5 cells were stained with *o*-dianisidine and H_2O_2 after a 5-day-incubation with the conditioned media from the individual clones. Magnification, X200.

presence of a neomycin resistance gene makes possible the selection of stable clones. Second, the CHO cells chosen for transfection are widely used for the expression of cloned genes, including secreted proteins and cell surface receptors, for both research and industrial application (Zaworski and Gill, 1993). Moreover, CHO cells have also been used to express recombinant goldfish activin B recently (Ge *et al.*, to be submitted).

The recombinant production of activin in mammals (Murata *et al.*, 1988; Schwall *et al.*, 1988; Mason *et al.*, 1989; Schmelzer *et al.*, 1990) has contributed significantly to the studies of activin in both mammalian and non-mammalian species. However, as the sequences of non-mammalian activin β subunits differ from the mammalian ones to different degrees (Ge *et al.*, 1993b; 1997a; Yam *et al.*, 1999b), problems of specificity and efficacy may arise when mammalian activins are used in the non-mammalian species. One of the solutions is to produce recombinant activin from the species of interest. Recently, recombinant goldfish activin B has been produced in our laboratory (Ge *et al.*, to be submitted) and is the vital source of activin for the ongoing studies in our group. The present study has also established two CHO cell lines (D1C7 and D1G10) that stably produce high levels of activin A with bioactivity. Their production of activin A was examined at both the transcriptional and secretion level. It is evident that the activity of recombinant activin A is well correlated with the level of mRNA expressed. It should be noted that most G418-selected CHO cell clones showed no sign of activin β_A mRNA expression, which might be due to the disruption of Gactβ_A cDNA or the promoter during integration. With the establishment of these stable cell lines (D1C7 and D1G10), we can propagate the cells for future large-scale production of recombinant activin A. The

purification may employ column chromatography and high performance liquid chromatography (HPLC) and the purification progress may be monitored by EDF-assay and Western blot analysis using specific antibodies for activin β_A.

In summary, the present study not only confirmed the functional identity of the goldfish activin β_A cDNA clone, thereby suggesting functional conservation of the activin A molecule across vertebrates in terms of EDF activity, but also established two CHO cell lines, which opens up the way for the recombinant production of goldfish activin A. This makes goldfish the only non-human vertebrate in which both recombinant activin A and B are available, which allows further comparative studies on the two activins in the species without problems of specificity and efficacy resulting from using heterologous molecules. This comparative information would not only enhance our understanding of activin functions in fish, but also vertebrates in general.

Chapter 4

Differential Regulation of Goldfish Gonadotropin (GTH-Iβ and GTH-IIβ) Gene Expression by Recombinant Goldfish Activin

4.1 Introduction

The differential regulation of gonadotropins (GTHs) has been well documented in mammals. In fish, two chemically distinct GTHs, GTH-I (αIβ) and GTH-II (αIIβ) have been purified and characterized in the chum salmon (*Oncorhynchus keta*) (Suzuki *et al.*, 1988a), coho salmon (*Oncorhynchus kisutch*) (Swanson *et al.*, 1991) and common carp (*Cyprinius carpio*) (van der Kraak *et al.*, 1992). The α subunit is common for the two GTHs, and the unique β subunit confers the biological specificity of the hormone. The duality of fish GTH has been further confirmed by molecular cloning of the -Iβ and -IIβ subunits in the chum salmon (Sekine *et al.*, 1989), killifish (*Fundulus heteroclitus*) (Lin *et al.*, 1992), masu salmon (*Oncorhynchus masou*) (Kato *et al.*, 1993), striped bass (*Morone saxatilis*) (Hassin *et al.*, 1995), gilthead seabream (*Sparus aurata*) (Elizur *et al.*, 1996), goldfish (*Carassius auratus*) (Yoshiura *et al.*, 1997) and Japanese eel (*Anguilla japonica*) (Yoshiura *et al.*, 1999). Although fish GTH-I and GTH-II exhibit similar biological activities *in vitro* (Suzuki *et al.*, 1988b; van der Kraak *et al.*, 1992), a phenomenon differing from the mammalian FSH (follicle stimulating hormone) and LH (luteinizing hormone), *in vivo* studies have shown that GTH-I and GTH-II have distinct patterns of expression and secretion during the reproductive cycle. The expression of GTH β subunits in salmonids has been shown to be closely associated

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with gonadal development. GTH-Iβ mRNA expression predominates in immature rainbow trout (*Oncorhynchus mykiss*) whereas high level of GTH-IIβ mRNA was found in the mature fish (Weil *et al.*, 1995). Similarly, in coho salmon, the high level of GTH-I during vitellogenesis is reduced before ovulation, while the level of GTH-II remains low during the early stages of the reproductive cycle and increases dramatically before maturation (Swanson, 1991). An increased expression of GTH-IIβ subunit during gonadal maturation was also evident in non-salmonid species such as the gilthead seabream (Meiri *et al.*, 1995). These differential expressions of GTH-I and GTH-II indicate that despite their similar activities *in vitro*, they are probably involved in controlling different phases of the fish reproductive cycle. To date, increasing evidence from different teleost species has lent support to this contention. In rainbow trout, it has been demonstrated that GTH-I but not GTH-II stimulates vitellogenin uptake by the growing oocytes both *in vitro* and *in vivo* (Tyler *et al.*, 1991). On the other hand, it is evident that GTH-II induces final oocyte maturation in the red seabream (*Pagrus major*) whereas GTH-I has no such an effect (Kagawa *et al.*, 1998). With the increasing understanding of the functions and expression patterns of GTH-I and GTH-II during the fish reproductive cycle, one of the questions to be answered is how the two GTHs are differentially regulated.

In mammals, three groups of factors, GnRH (gonadotropin-releasing hormone) from the hypothalamus, steroids from the gonads and activin/inhibin family of growth factors, play important roles in the regulation of FSH and LH. Activin and inhibin are critically important in the differential regulation of the two gondadotropins, though both GnRH and gonadal steroids also have differential

actions on FSH and LH production (Shupnik *et al.*, 1996). Activin selectively stimulates FSH secretion (Ling *et al.*, 1986b; Vale *et al.*, 1986) and FSHβ mRNA expression (Carroll *et al.*, 1989, 1991; Weiss *et al.*, 1992, 1993, 1995) in cultured and perifused rat pituitary cells, but has little (Carroll *et al.*, 1991; Ling *et al.*, 1986b; Weiss *et al.*, 1993) or very slight stimulatory effect (Vale *et al.*, 1986) on LH. *In vivo* studies using carotid catheters also indicate that activin contributes to the maintenance of FSHβ mRNA levels in intact rats (Besecke *et al.*, 1996).

In fish, although the effects of GnRH, neurotransmitters such as dopamine, and gonadal steroids on GTHs, particularly GTH-II, have been extensively studied (Habibi and Huggard, 1997; Peter and Yu, 1997; Sohn *et al.*, 1998), the information about activin involvement in the regulation of the two GTHs is very limited. Porcine activin A has been shown to stimulate goldfish GTH-II release from perifused pituitary fragments (Ge *et al.*, 1992) and both activin β_A and β_B have been identified in the goldfish by immunocytochemistry (Ge *et al.*, 1993a; Ge and Peter, 1994) and molecular cloning (Ge *et al.*, 1993b; Ge *et al.*, 1997a). These demonstrate the presence of activin in the goldfish and its regulation of GTH(s) in the species. However, due to the limited supply of activin and the lack of specific RIA (radioimmunoassay) for goldfish GTH-I, studies on the differential regulation of the two GTHs by activin at the secretion level have been hampered.

Recently, cDNAs for both the GTH-I and GTH-II β subunits have been cloned from the goldfish (Yoshiura *et al.*, 1997). This, together with the recombinant goldfish activin B recently produced in our laboratory (Ge *et al.*, to be submitted),

make possible the investigation of the involvement of activin in the differential regulation of GTH-I and GTH-II in the goldfish at the transcriptional level.

The present study aims at investigating the effects of recombinant activin B on goldfish pituitary GTH-Iβ and -IIβ mRNA expression using a well-established static culture system (Chang *et al.*, 1990; Levavi-Sivan and Yaron, 1992). This represents the first of this kind in fish and provides important information on activin involvement in the differential regulation of the two GTHs in non-mammalian species. A preliminary test on the effects of recombinant goldfish activin A will also be made using the culture medium from the activin A-producing CHO cells obtained from the last part.

4.2 Materials and Methods

All chemicals were obtained from Sigma (St. Louis, OH), enzymes from Promega (Madison, WI), and media for cell culture from Gibco BRL (Gaithersburg, MD) unless otherwise specified.

4.2.1 Animals

Common goldfish (*Carassius auratus*, body weight 30-60 g) of mixed sex were purchased from local market and acclimated in flow-through aquaria (1,000 litre) at 22°C with a photoperiod of 14 light:10 dark for at least one week before experiment. The fish were fed twice a day with commercial fish food during the period and were anaesthetized with tricane methanesulphonate before handling.

4.2.2 Drug treatment

Recombinant goldfish activin B (rgfActB) was prepared in our laboratory (Ge *et al.*, to be submitted) by expressing the protein in the Chinese hamster ovary (CHO) cells followed by purification from the medium according to Schmelzer *et al.* (1990). The activity of the purified protein [in 50 mM Tris-HCl and 150 mM NaCl (pH 7.4)] was monitored by EDF (erythroid differentiation factor)-assay using F5-5 cells, a mouse leukaemia cell line that responds specifically to activin by differentiating into haemoglobin-producing cells (Eto *et al.*, 1987). One unit (U) of rgfActB is defined as the amount per ml that induces a half-maximal differentiation (ED_{50}) of F5-5 cells in the bioassay (Schmelzer *et al.*, 1990), which is equivalent to 7 ng/ml of recombinant human activin A. Recombinant goldfish activin A (rgfActA) was produced by expression of the protein in CHO cells as described in the last chapter (Yam *et al.*, 1999b). Recombinant human follistatin (rhFS) was obtained from NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases) through the National Hormone and Pituitary Program. This product was reconstituted in 0.1 N acetic acid at a concentration of 1 μg/μl. The drugs were diluted with the culture medium immediately before application. For dose-response studies, pituitary cells were treated with different concentrations of rgfActB (0, 0.5, 1, 2, 5.5, 11, 55 and 110 U/ml) for 48 hours. For rgfActA test, cells were treated as follows: control, 50 μl of medium from pBK-transfected CHO cells and rgfActA, 50 μl of medium from pBK/Gactβ_A-transfected CHO cell clone D1C7 for 48 hours. For FS blockade experiment, cells were treated as follows: control (vehicle), rgfActB (5.5 U/ml), rgfActB (5.5 U/ml) + rhFS (350 ng/ml), and rhFS (350 ng/ml) for 48 hours.

4.2.3 Primary culture of dispersed pituitary cells

The pituitary cells were enzymatically dispersed and cultured according to Chang *et al.* (1990) and Levavi-Sivan and Yaron (1992) with modifications. Briefly, pituitaries collected from decapitated fish were maintained in Medium 199 with Earle's salt (M199) before cell dispersion. The pituitaries were washed three times with HBSS (Hank's balanced salt solution, 8 g NaCl, 0.4 g KCl, 0.014 g $CaCl_2$, 0.05 g Na_2HPO_4, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$ and 1 g glucose per litre) containing 20 mM HEPES, 0.3% BSA and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under aseptic conditions. The washed pituitaries were minced into fragments of about 0.5 mm^3 in size and washed with HBSS. The pituitary fragments were collected at the bottom of a 50 ml tube by centrifugation (1,200 rpm for 5 min) and treated with digestion solution (0.25% trypsin, 0.01% DNase II, 0.3% BSA in HBSS) at 28°C for 1 hour with gentle agitation. After trypsin/DNase digestion, fragments and cells were collected by centrifugation and stop solution (0.25% trypsin inhibitor, 0.01% DNase II and 0.3% BSA in Ca^{2+}-free HBSS) was added. The fragments were washed twice with washing solution (2 mM EDTA; 0.3% BSA in Ca^{2+}-free HBSS). The cells were dispersed mechanically by repeated pipetting in dispersion solution (0.002% DNase II, 0.3% BSA in Ca^{2+}-free HBSS). The dispersed cells were filtered through a 40 μm nylon mesh and resuspended in Ca^{2+}-free HBSS for the determination of cell yield and viability by trypan blue exclusion test. The cells were cultured in M199 with 10% FBS (Hyclone Laboratories, Logan, UT) and antibiotics at a density of $2\text{-}5\times10^5$ cells/0.5 ml/well in 24-well plates (Falcon, Franklin Lakes, NJ). The plates were precoated with poly-D-lysine to enhance cell attachment. After a 24-hour-preincubation at 28°C with 5% CO_2, the medium was changed and drug treatment initiated.

4.2.4 Southern blot analysis

cDNA clones of GTH-Iβ and GTH-IIβ (Yoshiura *et al.*, 1997) were digested with *Eco*RI to separate the inserts from the plasmids. One microgram of the digested plasmids were electrophoresed in 1% agarose gel and blotted onto positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) using VacuGene XL System (Pharmacia, Uppsala, Sweden). The blotted membrane was then UV-crosslinked at 150 mJ with GS GeneLinker (Bio-Rad, Hercules, CA). GTH-Iβ and GTH-IIβ cRNA probes were labelled with digoxigenin (DIG) by *in vitro* transcription using 1 μg linearized DNA, 1X DIG-RNA Labelling Mix (Boehringer Mannheim), 40 U T_3 RNA polymerase and 10 mM DTT in 1X Transcription Buffer. The nylon blot was hybridized overnight with the individual probe (25 ng/ml) at 50°C in hybridization solution containing 5X SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent (Boehringer Mannheim) and 50% formamide after a 2-hour-prehybridization at 50°C in hybridization solution without the probe. After hybridization, the blots were washed twice at room temperature with 2X SSC/0.1% SDS for 5 min each and twice at 65°C with 0.5X SSC/0.1% SDS and once with 0.1X SSC/0.1% SDS for 15 min each. Detection was performed using the Chemiluminescence Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, the membrane was blocked with blocking solution [1:10 w/v Blocking Reagent/maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5)] at room temperature for 1 hour after being equilibrated in washing buffer [maleic acid buffer, pH 7.5 and 0.3% w/v Tween 20]. The blocked membrane was incubated with anti-DIG antibodies (1:10,000) at room temperature for 30 min, washed twice in washing buffer for 15 min each and equilibrated in detection buffer (100 mM Tris-HCl and 100 mM NaCl, pH 9.5). Substrate solution (1:100 v/v

CSPD/detection buffer) was applied to the membrane followed by exposure to the X-ray film.

4.2.5 Isolation of total RNA

Total RNA was isolated from the pituitaries and cultured pituitary cells using TRI Reagent (Molecular Research Centre, Inc, Cincinnati, OH) according to the manufacturer's protocol. For RNA isolation from the tissue: One milligram of pituitary was homogenized in 1 ml of TRI Reagent and the homogenate was incubated at room temperature for 5 min. Then, two hundred microlitres of chloroform was added and the mixture was incubated at room temperature for 10 minutes. The aqueous layer was separated from the organic layer by centrifugation, and transferred to a fresh microtube. Half a millilitre of isopropanol and 0.5 μl of glycogen (20 mg/ml) were added for precipitation. After a 10-min-incubation at room temperature, the total RNA was pelleted, washed with 75% ethanol and dissolved in formamide. For the cultured cells: Cells were treated with 0.5 ml of TRI reagent, transferred to a fresh microtube and incubated at room temperature for 5 min. The following procedures were the same as those for the RNA isolation from the tissue except that all the reagents used were halved. The isolated RNA was stored at –20°C until dot blot analysis.

4.2.6 Northern blot analysis

Total RNA was electrophoresed in 1.1% formaldehyde denaturing gel [1.1% agarose in 1X MOPS (40 mM MOPS, 10 mM NaOAc and 1 mM EDTA, pH 7.0) and 2.2 M formaldehyde], blotted onto positively charged nylon membrane, UV-crosslinked, hybridized and detected by chemilunminescence as described above.

4.2.7 Dot blot analysis

Each RNA sample was divided in 20:4:1 for the detection of GTH-Iβ, GTH-IIβ and β-actin, respectively. The RNA samples were denatured at 68°C for 15 minutes in denaturing solution (50% formamide in 1X SSC) and blotted onto positively charged nylon membrane (Boehringer Mannheim) using Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Briefly, the membrane was prewetted with 10X SSC and placed onto the gasket. The apparatus was assembled and vacuum was applied. The wells were washed with 10X SSC followed by loading of the denatured samples in four volumes of 20X SSC. After the samples were vacuum-blotted, the wells were washed twice with 10X SSC. The blotted membrane was then removed, UV-crosslinked, hybridized and detected by chemilunminescence as described above. Multiple exposures were always made from the same blots with different exposure times. The image without significant sign of over exposure was used for quantification by densitometry.

4.2.8 Data analyses

For dot blot experiments, samples were obtained from at least triplicate wells and most of the experiments were repeated at least twice to ensure reproducibility of the results. Signals on the X-ray film was quantified by scanning densitometry using Densitometer 690 (Bio-Rad) and the software Molecular Analyst (BioRad). β-actin was used as an internal control to normalize the results (Querat *et al.*, 1991; Weil *et al.*, 1995; Yoshiura *et al.*, 1997). Data were statistically analysed by Student's *t*-test or one-way ANOVA followed by Fisher's least significance difference (LSD) comparison. $P<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Probe specificity

The specificity of GTH-Iβ and -IIβ probes was examined using Southern blot analysis. Results showed that the two probes were specific under the specified hybridization conditions (Fig. 4-1), in agreement with that reported by Yoshiura *et al.* (1997) using radioactive probes. In Northern blot analysis (Fig. 4-2), a clean single band was observed for GTH-Iβ, GTH-IIβ, and β-actin, which makes possible the use of dot blot for quantitation. As shown in Fig. 4-2, a dramatic difference was observed in the expression level of the two GTH subunits, with GTH-Iβ mRNA level much less abundant than that of GTH-IIβ; hence, a larger quantity of RNA had to be used for the measurement of GTH-Iβ mRNA.

4.3.2 Effects of goldfish activin on pituitary GTH-Iβ and -IIβ mRNA expression

To investigate the effects of activin B on the steady-state levels of the two GTH β subunits, pituitary cells were preincubated for 24 hours followed by treatment with different doses of rgfActB for 48 hours. As shown in Fig. 4-3, GTH-IIβ mRNA level decreased dramatically in response to activin in a dose-dependent manner with maximal effect at ~5.5 U/ml and ED_{50} ~0.55 U/ml. These values are comparable to the stimulatory effects reported for other actions of activin (Attardi and Miklos, 1990; Gonzalez-Manchon *et al.*, 1991; Gregg *et al.*, 1991; Vale *et al.*, 1986; Yu *et al.*, 1987). However, when the concentration of activin was further increased, the inhibitory effect diminished also in a dose-dependent manner, thus exhibiting a

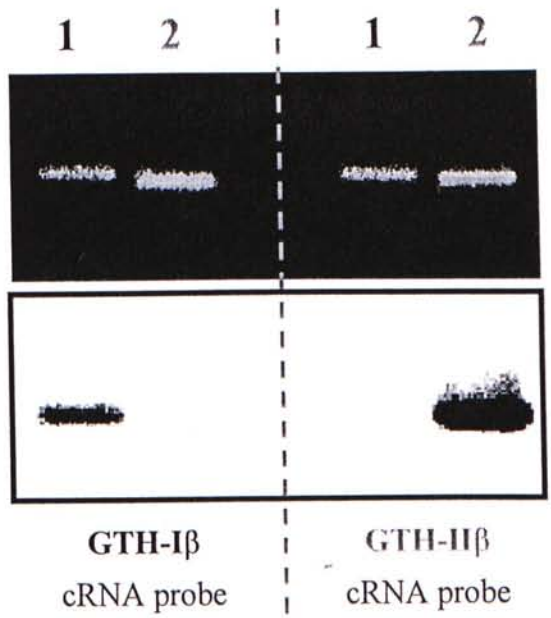


Fig. 4-1 Southern blot analysis for the specificity of GTH-Iβ and -IIβ probes. GTH-Iβ and -IIβ cDNA were separated from the vectors by enzyme digestion, electrophoresed in lane 1 and 2 respectively (*upper panel*), blotted onto nylon membranes, and hybridized with GTH-Iβ and GTH-IIβ probes as shown (*lower panel*). Results show that the probes used were specific with no crossreactivity with the other.

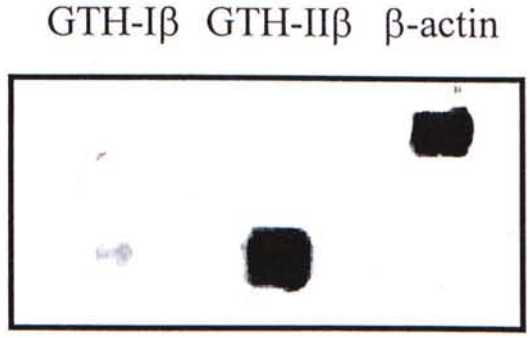


Fig. 4-2 Northern blot analysis. Fifteen, five and five micrograms of total RNA from the pituitary were electrophoresed, blotted onto nylon membrane and hybridized with GTH-Iβ, GTH-IIβ and β-actin probes, respectively as indicated.

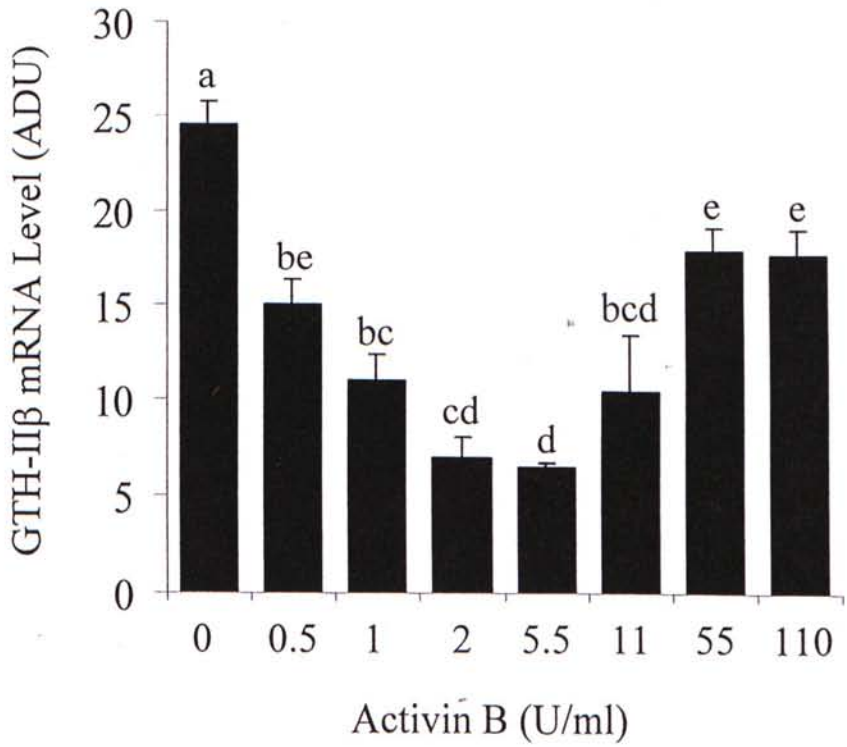


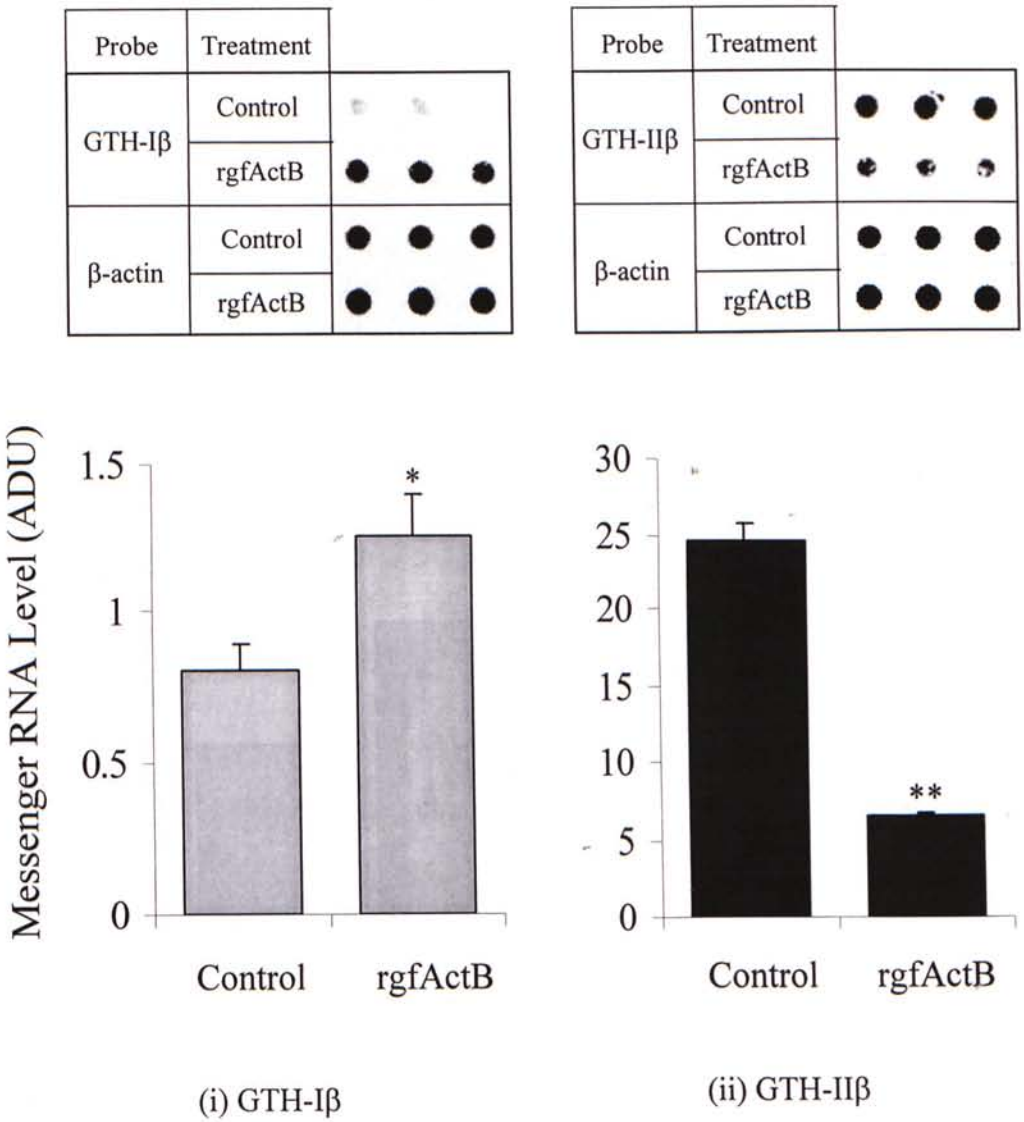
Fig. 4-3 Dose-response of GTH-IIβ mRNA expression to rgfActB. Results (normalized with β-actin) represent mean±SEM of 3-4 determinations from a typical experiment representing 3 independent experiments. Different letters represent data with significant difference ($P<0.05$). ADU, arbitrary densitometric unit.

biphasic response. This inhibitory effect has been confirmed consistently in repeated experiments. Owing to the low level of mRNA expression (Fig. 4-2), it was difficult to construct a full dose-response curve for GTH-Iβ; therefore, the dose that caused maximal inhibition of GTH-IIβ (5.5 U/ml), was used to investigate the effect of rgfActB on GTH-Iβ expression. As shown in Fig. 4-4, rgfActB showed opposite effects on the two GTH β subunits. It decreased GTH-IIβ mRNA expression while stimulated that of GTH-Iβ. The mRNA level of β-actin remained unchanged and was used to normalize the data for statistical analyses.

Preliminary results using activin A-containing medium indicated that rgfActA also had differential effects on the two GTHs. Like rgfActB, rgfActA stimulated GTH-Iβ but suppressed GTH-IIβ mRNA expression (Fig. 4-5). Further experiments using purified rgfActA and rgfActB would confirm this and shed light on whether these two forms of activin have any differences in potency in regulating GTH expression.

4.3.3 Blockade of activin effects by follistatin

To demonstrate that the actions of rgfActB on the steady-state levels of pituitary GTH β subunits were specific, experiments were performed in which rgfActB (5.5 U/ml) was applied together with rhFS, a protein that binds specifically to activin and neutralize its biological activities (Hashimoto *et al.*, 1997; Kogawa *et al.*, 1991; Nakamura *et al.*, 1990). As shown in Fig. 4-6, both the inhibitory effect on GTH-IIβ and the stimulatory effect on GTH-Iβ mRNA were completely abolished by rhFS (350 ng/ml), thereby confirming the specificity of the novel effects of rgfActB on the expression of the two GTH β subunits. It should be noted that, though not



(i) GTH-Iβ

(ii) GTH-IIβ

Fig. 4-4 Effects of rgfActB (5.5 U/ml) on goldfish (i) GTH-Iβ and (ii) GTH-IIβ subunit mRNA expression. *Upper panel*: Dot blot images from a representative experiment. *Lower panel*: Quantified data (normalized with β-actin) from a typical experiment representing three independent experiments. Values are mean±SEM of 3 determinations. *P<0.05; **P<0.0001. ADU, arbitrary densitometric unit.

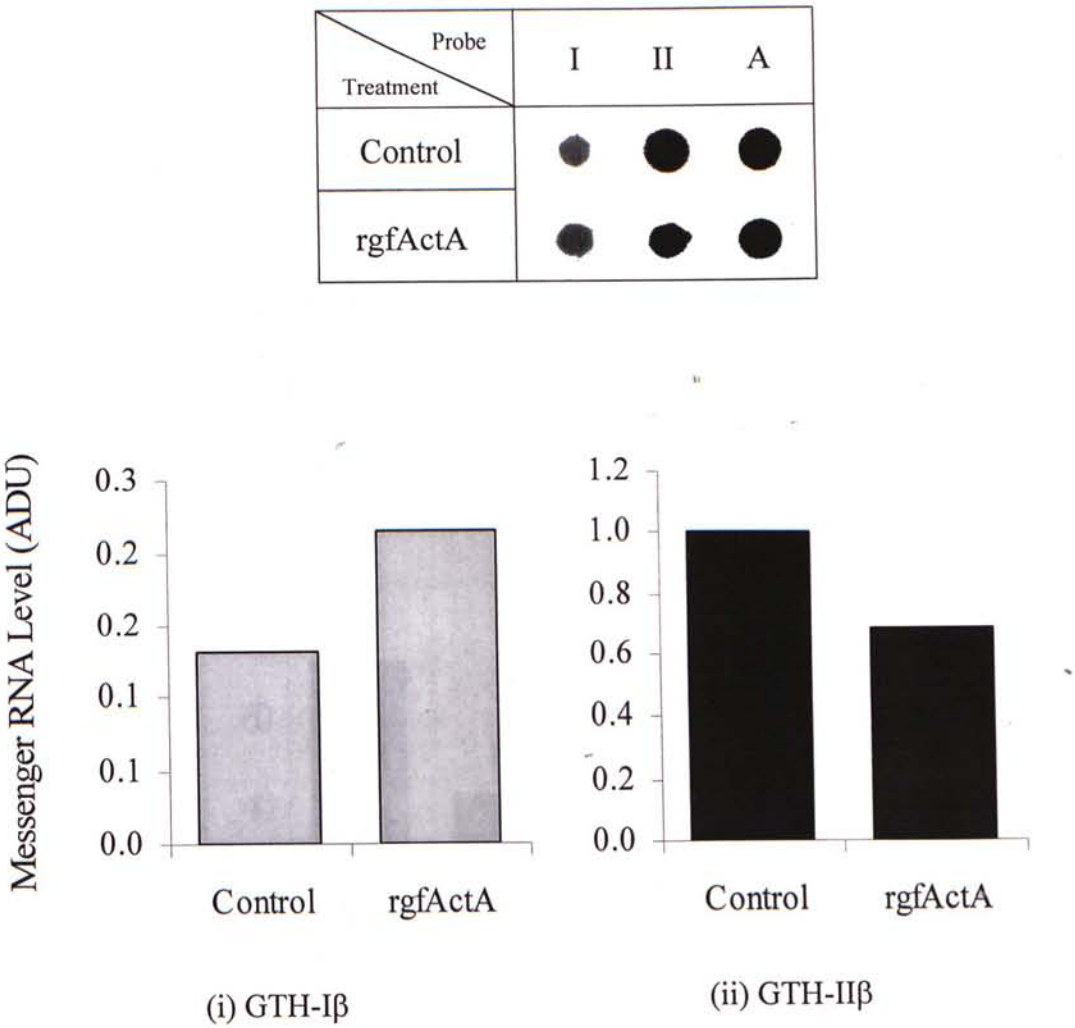


Fig. 4-5 Effects of rgfActA on goldfish (i) GTH-Iβ and (ii) GTH-IIβ subunit mRNA expression. *Upper panel*: Dot blot image. *Lower panel*: Quantified data (normalized with β-actin). I, GTH-Iβ; II, GTH-IIβ; A, β-actin; control, culture medium (50 μl) from pBK-transfected CHO cells; rgfActA, culture medium from pBK/Gactβ$_A$-transfected CHO cell clone D1C7 (Yam *et al.*, 1999b); ADU, arbitrary densitometric unit.

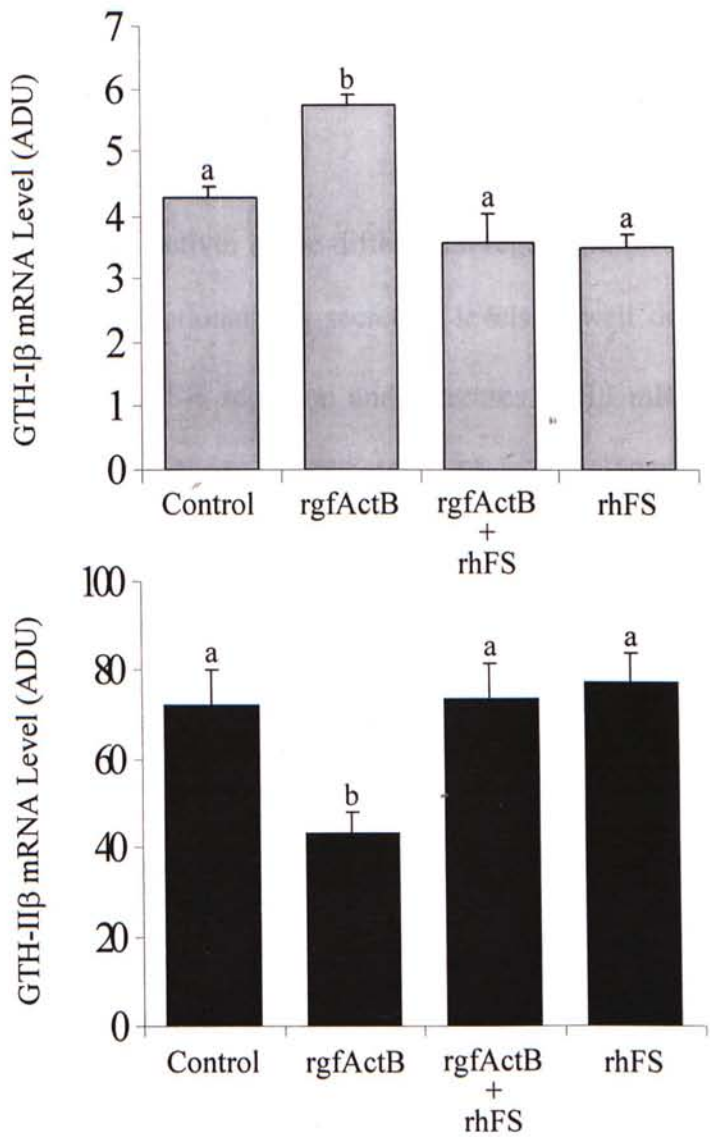


Fig. 4-6 Blockade of rgfActB (5.5 U/ml) effects by rhFS (350 ng/ml). *Upper panel*: Effect on GTH-Iβ mRNA. *Lower panel*: Effect on GTH-IIβ mRNA. Data (normalized with β-actin) represent mean±SEM of 3 determinations. Different letters represent data with significant difference ($P<0.05$). ADU, arbitrary densitometric unit.

statistically significant, the application of rhFS alone decreased the level of GTH-Iβ mRNA and increased that of GTH-IIβ mRNA, an effect opposite to that exerted by activin, suggesting that endogenous activin may be present in the goldfish pituitary.

4.4 Discussion

The involvement of activin in the differential regulation of mammalian FSH and LH at both the transcriptional and secretion levels is well documented. Activin specifically stimulates FSH secretion and increases FSHβ mRNA expression with little effect on LH (Carroll *et al.*, 1989, 1991; Ling *et al.*, 1986b; Vale *et al.*, 1986; Weiss *et al.*, 1992, 1993, 1995). Although activin has also been demonstrated in a variety of non-mammalian species, very little is known about its regulation of pituitary gonadotropin production. The immunoreactivities for activin subunits (Ge and Peter, 1994) and the expression of β_B mRNA (Ge *et al.*, 1997a) have been demonstrated in the goldfish pituitary. This, together with our recent demonstration of the expression of activin receptors in the pituitary (Ge *et al.*, unpublished), strongly suggests paracrine and autocrine roles for activin in the regulation of goldfish pituitary functions. The present study demonstrated that activin regulated goldfish GTH expression *in vitro*, which represents the first of this kind in lower vertebrates. When applied to the cultured goldfish pituitary cells, rgfActB significantly increased the mRNA level of GTH-Iβ, an effect consistent with that in mammals if GTH-I is considered as an FSH-like fish gonadotropin as recently suggested (Swanson and Dittman, 1997; Tyler *et al.*, 1997). However, the most striking observation from the present study was that activin B dramatically decreased GTH-IIβ mRNA level in a dose-dependent manner. The opposing effects of activin B on goldfish GTH-Iβ and -IIβ expression appear to be analogous to those in sheep,

in which activin has been shown to stimulate GnRH-induced FSH secretion but inhibit LH release (Muttukrishna and Knight, 1991). Both the stimulatory effect on GTH-Iβ and inhibitory effect on GTH-IIβ mRNA expression by activin could be abolished by recombinant human follistatin, a specific activin-binding protein (Hashimoto *et al.*, 1997; Kogawa *et al.*, 1991; Nakamura *et al.*, 1990), therefore confirming the specificity of the novel activin activities. The effects of rhFS alone (decrease in GTH-Iβ mRNA and increase in GTH-IIβ mRNA basal level), though statistically not significant, suggest possible presence of endogenous activin and its potential autocrine/paracrine roles within the goldfish pituitary. These are supported by the existing data that activin subunits (Ge and Peter, 1994; Ge *et al.*, 1997a) and receptors (Ge *et al.*, unpublished) are present and expressed in the goldfish pituitary. Another interesting observation is that the powerful inhibitory effect of activin B on GTH-IIβ mRNA level was attenuated when activin concentration was further increased beyond the maximal inhibitory dose. This kind of biphasic response has also been reported for activin stimulation of GnRH receptor in the rat (Ferandez-Vazquez *et al.*, 1996). However, the mechanism for the loss of effects at the high concentration range is unknown and remains an interesting issue for future investigation. Studies are now underway in our laboratory to investigate the mechanism by which activin B exerts its action to increase GTH-Iβ but decrease GTH-IIβ mRNA levels. Preliminary results using activin A-containing medium showed that rgfActA also had opposite effects on the two GTHs. It stimulated GTH-Iβ but inhibited GTH-IIβ mRNA expression in a manner similar to rgfActB. However, whether the two forms of activin have the same potency in terms of the differential regulation of the two GTHs awaits further elucidation after the purification of the protein.

The inhibitory effect of activin B on GTH-IIβ expression somehow contradicts with our previous observation that purified porcine activin A stimulated goldfish GTH-II secretion from the pituitary fragments in perifusion (Ge *et al.*, 1992). The discrepancy could be attributed to several reasons. First, goldfish activin B instead of heterologous mammalian activin A was used in the present study. Second, the dispersed pituitary cells were treated with activin B for 48 hours, much longer than those used before. Third, activin may have different effects on GTH-II production at the transcriptional and secretion levels. Studies involving measurement of GTH-II secretion are now underway in our laboratory to clarify this issue.

In salmonids, GTH-I and GTH-II were reported to have distinct pattern of expression and secretion during the reproductive cycle (Breton *et al.*, 1998; Swanson, 1991; Weil *et al.*, 1995). GTH-I level is high during the vitellogenic stage but drops dramatically when the oocyte become fully-grown. In contrast, GTH-II level remains low during the period of vitellogenesis, but increases sharply before ovulation. These lines of evidence indicate that GTH-I and GTH-II play distinct roles during the reproductive cycle. It is likely that GTH-I is responsible for promoting oocyte growth (vitellogenesis) whereas GTH-II is mainly involved in inducing final oocyte maturation and ovulation. This hypothesis is now supported by increasing functional evidence from both salmonids and other teleost species. GTH-I promotes vitellogenin incorporation into the growing oocytes in the rainbow trout, whereas GTH-II has no such an effect (Tyler *et al.*, 1991). On the other hand, it was recently shown that in the red seabream, it is GTH-II but not GTH-I that induces oocyte maturation (Kagawa *et al.*, 1998). Interestingly, a recent study

demonstrated that the postovulatory plasma profiles of GTH-I and GTH-II in the rainbow trout depend on whether the ovulated eggs are released or remain in the ovarian cavity. The release of the ovulated eggs by stripping is followed by a significant increase in GTH-I levels and decline in GTH-II levels. However, if the ovulated eggs remain in the ovarian cavity, the secretion of GTH-II increases without significant changes in GTH-I levels, which prevents the recruitment of the new follicles for the next cycle of development. These observations, together with the evidence that GnRH has no stimulatory effect on GTH-I secretion, have led to the hypothesis that there might be certain unknown ovarian factors that have antagonistic effects on the two GTHs (Breton *et al.*, 1998). It is obvious that from these studies, the expression and secretion of the GTHs in fish are well timed and tightly controlled during the reproductive cycle. Although the regulatory mechanisms behind the differential expression and secretion remain unclear, the present finding that activin B promotes GTH-Iβ while inhibits GTH-IIβ expression strongly suggests that activin may play an important role in controlling and coordinating the production of the two gonadotropins in fish. More studies are needed to elucidate the physiological relevance of activin in this aspect.

In summary, the present study demonstrates, for the first time, that activin has opposite effects on GTH-Iβ and GTH-IIβ mRNA expression. Recombinant goldfish activin stimulates GTH-Iβ but suppresses GTH-IIβ mRNA levels *in vitro*. This finding not only contributes to our understanding of the mechanisms that control the temporal expression pattern of the two GTHs during fish reproductive cycle, but also provides important information on the evolution of the mechanisms that control GTH production in vertebrates. The novel opposite effects of activin on the two

goldfish GTHs also make goldfish a unique and attractive vertebrate model for activin studies. Furthermore, studies on the mechanism by which activin differentially regulates the two GTHs in fish will provide useful information for developing novel approaches to control and manipulate fish reproduction in aquaculture.

Chapter 5

General Discussion

5.1 Overview

Gonadadotropins (GTHs) are important regulators in vertebrate reproduction. They modulate the critical events of steroidogenesis, gametogenesis, ovulation and spermiation throughout the reproductive cycle, thus working for the ultimate goal of the animals, the perpetuation of species. For this reason, research on GTHs has attracted much attention.

The duality of fish GTHs is evident as two chemically distinct GTHs (GTH-I and GTH-II) have been identified in different orders of teleosts including the eel, tilapia, salmonids and cyprinids (Elizur *et al.*, 1996; Hassin *et al.*, 1995; Kato *et al.*, 1993; Lin *et al.*, 1992; Sekine *et al.*, 1989; Suzuki *et al.*, 1988a, 1988b; Swanson *et al.*, 1991; van der Kraak *et al.*, 1992; Yoshiura *et al.*, 1997, 1999), either by molecular cloning or purification from natural sources. Both GTH-I ($\alpha I\beta$) and GTH-II ($\alpha II\beta$) are heterodimers consisting of a common α subunit and a unique β subunit for specific bioactivity of the hormone. Increasing evidence from different species show that the two GTHs have distinct patterns of expression throughout the reproductive cycle, with high level of GTH-I during early gonadal development, probably for controlling vitellogenesis; and high level of GTH-II during gonadal maturation, obviously for inducing ovulation and spermiation (Kagawa *et al.*, 1998; Meiri *et al.*, 1995; Swanson, 1991; Tyler *et al.*, 1991; Weil *et al.*, 1995). One important question brought up by these observations is how these two GTHs are differentially regulated.

The answer to this question is of great physiological importance and much of the ongoing research is pointing towards this direction. Based on the available information from the mammals, a possible candidate for the differential regulation of fish GTHs is activin.

Activin is a dimeric protein consisting of two β subunits. Three dimeric forms of activin have been purified from natural sources: activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$) and activin B ($\beta_B\beta_B$) (Ling *et al.*, 1985, 1986a, b; Vale *et al.*, 1986, 1988; Ying *et al.*, 1988). Activin was first identified in a fraction of the follicular fluid that selectively stimulates FSH secretion, but has no effect on LH (Ling *et al.*, 1985, 1986b; Vale *et al.*, 1986). Later, activin was demonstrated to have wide tissue distribution and be involved in a vast array of physiological and developmental events (DePaolo *et al.*, 1991; Menuier *et al.*, 1988). In many cases, it acts in an autocrine/paracrine manner (Chen, 1993). The contribution of activin in the differential regulation of FSH and LH, the homologues of fish GTH-I and GTH-II, respectively, has been demonstrated in both *in vivo* and *in vitro* studies at the transciptional, posttranscriptional and secretion level (Carroll *et al.*, 1991; Dalkin *et al.*, 1994; Halvorson *et al.*, 1994; Weiss *et al.*, 1992, 1993, 1995).

In fish, subunits of activin have also been demonstrated in a number of species including the goldfish by immunocytochemistry or molecular cloning (Ge and Peter, 1994; Ge *et al.*, 1993a, 1993b, 1997a; Nagahama, 1994; Tada *et al.*, 1998; Wittbrodt and Rosa, 1994). Full-length cDNA for activin β_B has been cloned in the goldfish (Ge *et al.*, 1997a), zebrafish (Wittbrodt and Rosa, 1994) and Japanese eel (Nagahama, 1994). However, no full-length sequence has yet been published for

activin β_A in any fish species. Previous reports have shown that mature goldfish activin β_A, which shares about 80% amino acid sequence homology with that of mammals, is more variable than its β_B counterpart (93-95%) (Ge *et al.*, 1993b). Therefore, cloning of the full-length activin β_A is of importance for comparative studies of the functions of activin A and activin B.

On the other hand, porcine activin A was shown to stimulate GTH-II release in perifused goldfish pituitary cells (Ge *et al.*, 1992), suggesting that activin is involved in the regulation of fish GTHs. However, due to the limited supply of activin and the lack of specific RIA for goldfish GTH-I, studies on the differential regulation of the two GTHs by activin at the secretion level have been hampered.

Recently, cDNAs for both the GTH-Iβ and -IIβ subunits have been cloned from the goldfish (Yoshiura *et al.*, 1997) and recombinant goldfish activin B is now being produced in our laboratory (Ge *et al.*, to be submitted). These make possible the investigation of the differential regulation of the two GTHs by activin at the transcriptional level.

5.2 Contribution of the Present Research

5.2.1 Cloning of full-length goldfish activin β_A cDNA

In the present study, a full-length cDNA coding for activin β_A (Gactβ_A) has been isolated from the goldfish. This is the first published sequence of full-length activin β_A cDNA in a teleost (Yam *et al.*, 1999b; GenBank accession number AF169032). The predicted 404 amino acid precursor shows about 80% and 60% sequence identity to that of mammals over the mature region and the entire precursor,

respectively. The mature peptide shares 100% sequence identity with the genomic clone previously obtained (Ge *et al.*, 1993b), but the identity at the nucleotide level is only about 70%. Together, these demonstrate the presence and expression of two isoforms of activin β_A in the species. Goldfish activin β_A has been shown to express in different goldfish tissues including ovary, testis, brain and liver (Yam *et al.*, 1999b), which is consistent with other studies in fish and mammals (Ge *et al.*, 1997a, b; Meunier *et al.*, 1988). Goldfish β_A (Yam *et al.*, 1999b) and β_B subunits (Ge *et al.*, 1997a) shares only 44% and 60% amino acid sequence identity over the entire precursor and the C-terminal mature region, respectively. This raises the question of whether there is any functional difference between the two subunits. To date, the question of why goldfish produces two similar activin β subunits with different degree of conservation, with β_B more conserved (more than 90% homology with the mammalian molecules) and β_A more variable (80%), remains unanswered and awaits further elucidation.

5.2.2 Establishment of stable cell lines for the recombinant production of goldfish activin A

The functional identity of the full-length activin β_A cDNA (Gactβ_A) has been confirmed by expressing the protein in Chinese hamster ovary (CHO) cells, followed by detection of its specific activin activity in an EDF(erythroid differentiation factor)-assay.

The present study has also established two CHO cell lines that produce high level of bioactive recombinant goldfish activin A at both the transcriptional and secretion levels (Yam *et al.*, 1999b). This, together with the recombinant goldfish activin B

recently produced in our laboratory (Ge *et al.*, to be submitted), make goldfish the only non-mammalian model in which both recombinant activin A and B are available. This would allow detailed comparative studies on their functions without problems of specificity and efficacy that may arise when using the heterologous mammalian molecules.

5.2.3 Differential regulation of goldfish gonadotropin (GTH-Iβ and GTH-IIβ) gene expression by recombinant goldfish activin

The present study presents the first piece of evidence for the differential regulation of GTH-I and GTH-II by activin. Recombinant goldfish activin has opposite effects on the two GTH β subunits. It stimulates GTH-Iβ but suppresses GTH-IIβ mRNA levels *in vitro* (Yam *et al.*, 1999a). This finding not only enhances our understanding of the mechanisms that control the temporal expression pattern of the two GTHs during fish reproductive cycle, but provides important information on the evolution of mechanisms that control GTH production in vertebrates. The novel opposite effects of activin on the two goldfish GTHs also make goldfish a unique and attractive model for activin studies.

A proposed model for the involvement of activin in the regulation of fish reproduction is given in Fig. 5. As demonstrated by Ge *et al.* (1997a) and Yam *et al.* (1999b), activin subunits are present in a number of tissues including the brain, pituitary, ovary, testis and liver in the goldfish, suggesting that activin may be involved in every regulatory level of reproduction. Besides, activin differentially regulates GTH-Iβ and GTH-IIβ gene expression (Yam *et al.*, 1999a), demonstrating

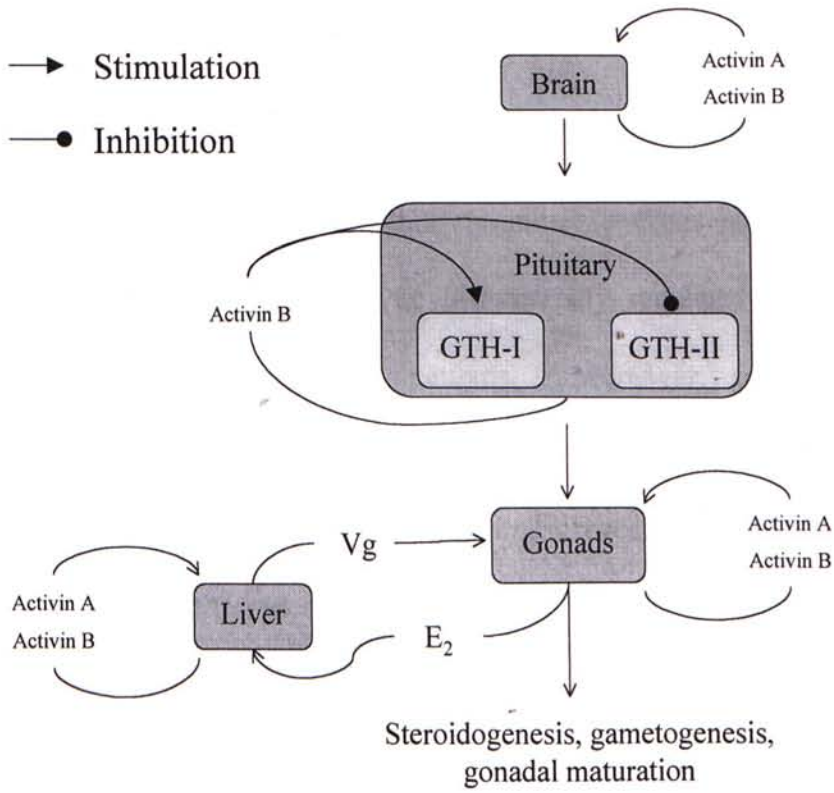


Fig. 5 Involvement of activin in the regulation of fish reproduction. E_2, oestradiol; Vg, vitellogenein.

that activin plays a critical role in regulating GTH expression throughout the fish reproductive cycle.

5.3 Future Research Direction

5.3.1 Activin studies

To understand why goldfish produces two similar activin β subunits, with one being more conserved than the other, promoter analysis should be performed. Activin β_A and β_B subunits may be differentially regulated and have different temporal and spatial expression patterns. Moreover, purification of both recombinant goldfish activin A and B will allow extensive biochemical and physiological characterization. This may in turn reveal if goldfish activin A and B have any functional differences.

5.3.2 Gonadotropin studies

As activin has differential regulation of the two GTH β subunit (GTH-Iβ and GTH-IIβ) mRNA expression, the next step is to identify possible activin-responsive element(s) in the promoters of the GTH-Iβ and -IIβ subunit genes. Detail promoter analysis will shed light on the mechanisms involved in the differential regulation. Besides, actinomycin D and cyclohexamide treatment should be performed to dissect out whether the differential regulation is on the transcriptional or posttranscriptional level. Moreover, attention should be paid to see if the two subunits are also differentially regulated at the secretion level if specific RIA or ELISA for both GTH-I and GTH-II could be developed.

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